The intake of polyunsaturated fatty acids and cardiovascular diseases

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The intake of polyunsaturated fatty acids and cardiovascular diseases

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Abstract

Background

Despite a large amount of research in the past decades, the role of polyunsaturated fatty acids (PUFA) in the prevention of coronary heart disease (CHD) and stroke is still debated. Inconsistent findings in epidemiological studies may be due to methodological limitations of dietary assessment, which could be overcome by using PUFA levels in blood as a biomarker of intake. This thesis investigates dietary intake and plasma levels of various n-6 and n-3 PUFA in relation to CHD and stroke within a population-based sample in the Netherlands.

Methods

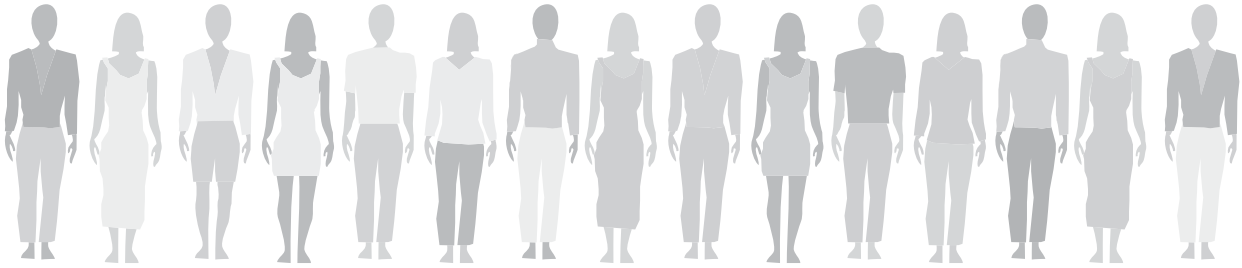
The associations of dietary intake of PUFA (assessed by food-frequency questionnaire) with incident CHD and stroke were examined in cohort studies. PUFA levels in plasma cholesteryl esters were measured in nested case-control studies. N-6 PUFA included linoleic acid and arachidonic acid and the n-3 PUFA included the marine-derived eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and the plant-derived alpha-linolenic acid (ALA). We used the "Monitoring Project on Cardiovascular Disease Risk Factors" and the "Monitoring Project on Risk Factors for Chronic Diseases" (MORGEN study), two large similar population-based cohorts with baseline measurements in 1987-1997 and follow-up for CHD and stroke incidence. Additionally, we performed a meta-analysis of prospective epidemiological studies on PUFA in cholesteryl esters and CHD risk.

Results

A 4-5 en% difference in linoleic acid intake was not associated with incident CHD, whereas plasma linoleic acid was inversely, but statistically non-significantly, associated with fatal CHD. In the meta-analysis, a 5% higher plasma linoleic acid level was related to a significant 9% lower CHD risk. Both ALA intake and status were not associated with CHD. The top quartiles of EPA-DHA (~250 mg/d) and fish intake (~1 fish meal/week) were related to a ~50% lower risk of fatal CHD compared to the bottom quartiles. However, this was not confirmed in plasma EPA-DHA. An ALA intake ≥1.1 g/d was associated with a 35-50% lower stroke incidence, compared with lower intakes. In women, but not in men, a significantly inverse relation was observed for EPA-DHA and fish intake with incident stroke, with a ~50% lower risk in the top quartile compared with the bottom quartile. Plasma PUFA levels were, however, not related to incident stroke.

Conclusion

The hypothesis of a beneficial effect of linoleic acid on CHD was confirmed in our biomarker study, but not in the study that used dietary intake data. For EPA-DHA, on the other hand, dietary intake was inversely related to fatal CHD and incident stroke, whereas cholesteryl ester EPA-DHA were not associated. The same applied to ALA intake in relation to incident stroke. Inconsistencies between PUFA intake and status with cardiovascular diseases could be attributed to the limited range of variation in PUFA intake in combination with measurement error.



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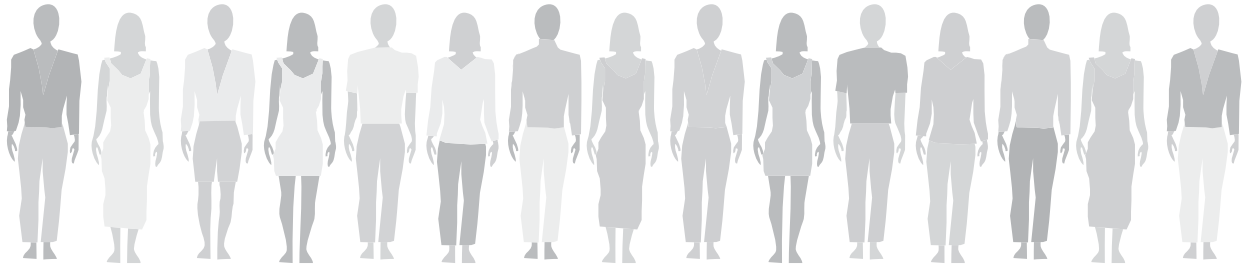

Introduction

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Introduction

Fatty acids in the diet can be classified as saturated fatty acids, monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) according to the number of their double bonds (**Figure 1.1**). Saturated fatty acids, which have no double bonds, are mainly derived from foods of animal origin such as meat and dairy, and also from a limited number of vegetable sources such as coconut oil. The main dietary MUFA is oleic acid, present in olive oil. *Trans*-fatty acids are unsaturated fatty acids (mainly MUFA) with a double bond in *trans*-configuration. *Trans*-fat in the diet originates from industrial hydrogenation used to produce semi-liquid and solid fats for the production of margarine, shortenings, and cookies and also from dairy and meat of ruminant animals.[1] Most dietary unsaturated fatty acids, however, have the *cis*-configuration.[1] This thesis focuses on (*cis*-) PUFA. These fatty acids with at least two double bonds, will be described in further detail below.

Polyunsaturated fatty acid intake

Dietary PUFA can be divided into n-6 (or omega-6) and n-3 (or omega-3) PUFA. N-6 and n-3 PUFA have a first double bond in the n-6 position or n-3 position, respectively, counted from

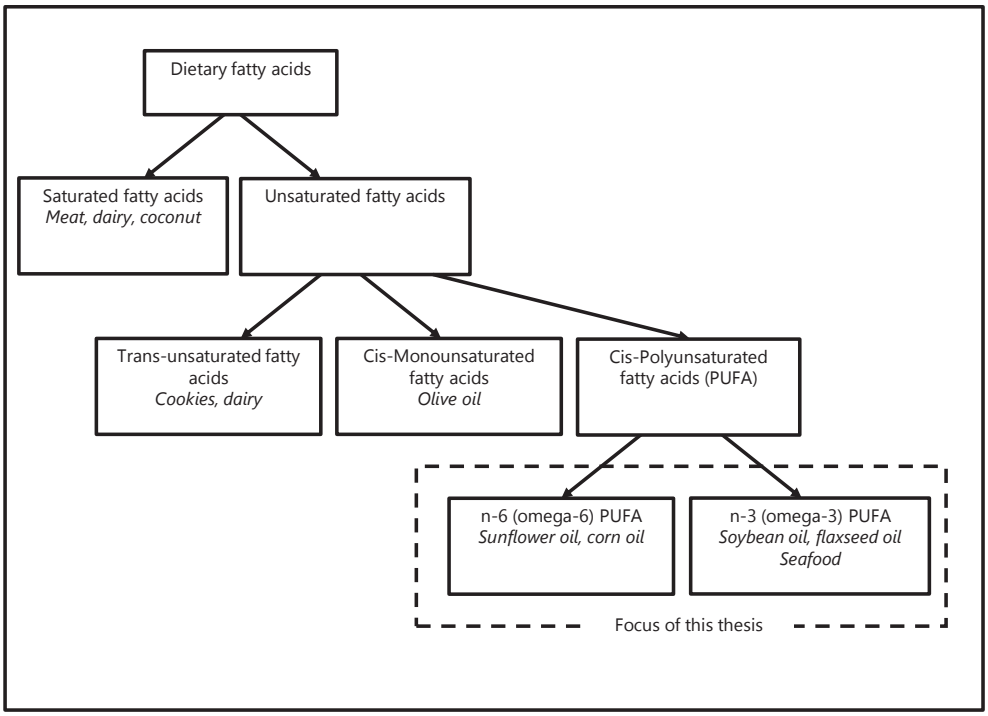

Figure 1.1 Types of dietary fatty acids.

Figure 1.2 Structures of linoleic acid (n-6) and alpha-linolenic acid (n-3).

the methyl end (**Figure 1.2**). N-6 PUFA currently contribute for 85-90% of total PUFA intake.[2] Two important n-6 PUFA are linoleic acid (C18:2n-6) and arachidonic acid (C20:4n-6). N-3 PUFA form the remaining 10-15% of total PUFA intake. N-3 PUFA can be divided into n-3 fatty acids from vegetable sources, such as alpha-linolenic acid (ALA; C18:3n-3) and n-3 fatty acids from marine sources, such as eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3).

Dietary sources of arachidonic acid include eggs and lean meat, such as poultry, organ meats, and fish,[3-5] but most of the arachidonic acid in the human body is derived from elongation of linoleic acid.[4,5] Linoleic acid, is the most abundant PUFA in the diet. It is mainly obtained from vegetable oils, such as sunflower oil and soybean oil.[6] Linoleic acid is an essential PUFA, which means that it must be provided by the diet. An intake of 2% of energy (en%) per day is enough to prevent deficiency.[7] Worldwide the daily intake ranges from 3 en% (India) to 10 en% (Israel).8,9 In the Netherlands, the average linoleic acid intake is 5-6 en% (~14 g/d).[10-12]

ALA is an essential fatty acid of the n-3 PUFA family and it is present in soybean, canola, and flaxseed oil, and in walnuts.[6] In the Netherlands, the average ALA intake in young adults was approximately 1.5 g/d (0.6 en%) in 2003.[13] Humans can convert ALA into the very-long-chain n-3 PUFA (EPA and DHA), although these conversions only take place to a limited extent. Conversion estimates are <8% for ALA to EPA and <1% for ALA to DHA.[14-16] Fish is the main source of EPA and DHA.[6] Other foods like meat and eggs contribute ~35% to the total intake of EPA and DHA.[2] The intake of fish and consequently EPA and DHA is low in the Netherlands. The general Dutch population consumed on average about 10 gram of fish per day in 1998.[11] The average EPA-DHA intake in young adults in 2003 was 94 mg/d.[13] Because these amounts were based on two 1-day dietary histories or two 24h recalls and because fish is not part of the everyday diet in the Netherlands, consumption is likely to be underestimated. The Dutch Fish Product Board estimated that in 2010 the Dutch consumed on average 10 g of fish per day at home and a similar additional amount outside the house.[17] In the European Prospective Investigation into Cancer and Nutrition, the Dutch had the lowest fish consumption, together with the Germans, whereas Mediterranean and Scandinavian populations consumed much more fish.[18]

Polyunsaturated fatty acid status and biomarkers of intake

After ingestion of fatty acids, various metabolic steps take place, such as β-oxidation, desaturation, and elongation (**Figure 1.3**). The Δ5- and Δ6-desaturases are key enzymes in PUFA metabolism that catalyse the conversion of linoleic acid into arachidonic acid and that of ALA into EPA. The activity of Δ6-desaturase is the rate-limiting step in the PUFA biosynthesis pathway. Because linoleic acid and ALA share the same metabolic pathway, they compete for the desaturase enzymes. In the body, fatty acids function as storage unit for energy, as structural unit in membranes, and as precursor to eicosanoids.[3] Arachidonic acid and EPA are precursors for eicosanoids such as prostaglandins and thromboxanes.[4] The arachidonic acid derived eicosanoids generally have proinflammatory and proaggregatory effects whereas EPA-derived eicosanoids have opposite effects.[19]

Ingested fatty acids can be measured as free fatty acids in serum (or plasma), as components of triglycerides, phospholipids, cholesteryl esters, erythrocyte membranes, platelets or in adipose

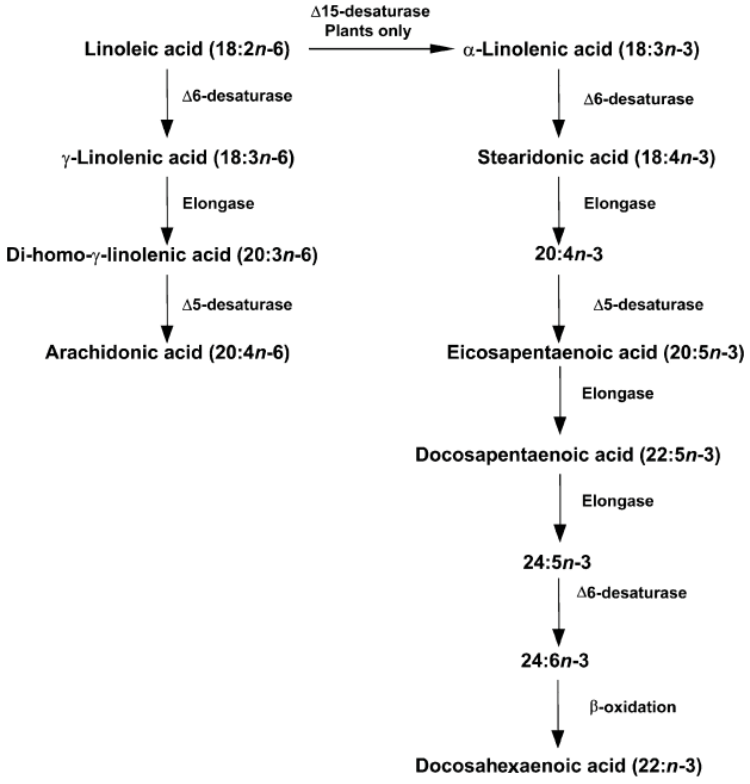

Figure 1.3 Pathway of the conversion of linoleic acid and alpha-linolenic acid to their longer chain derivates.[5]

tissue from various sites.[3] The amount of specific fatty acids can vary between the tissue fractions. Cholesteryl esters are found in serum lipoproteins and reflect dietary intake of PUFA during the previous weeks.[20,21] Whole serum, serum fractions, and erythrocytes also reflect a relatively short-term intake (between days and months). In long-term observational studies, adipose tissue is considered the best choice to assess habitual fatty acid intake, because it reflects the intake of fatty acids during the previous months to years.[21,22] However, in observational studies blood tissue is most widely used because of its accessibility and the assumption that individuals do not make drastic short-term diet changes.[22]

Polyunsaturated fatty acids and cardiovascular diseases

According to the classic Diet-Heart Hypothesis,[23] saturated fatty acids increase serum (LDL) cholesterol, high serum cholesterol causes atherosclerosis, and atherosclerosis results in coronary heart disease (CHD). CHD and stroke are the main types of cardiovascular disease (CVD). Stroke can be classified into ischemic stroke, which is caused by obstruction of a brain blood vessel, and haemorrhagic stroke, caused by bleeding into the brain. Ischemic stroke is the most common stroke type in Western countries.[24] Although CHD and ischemic stroke partly have similar etiologies, they may be affected differently by fatty acid intake.[24] For example, prospective cohort studies have not found significant associations between n-6 PUFA intake and ischemic or hemorrhagic stroke or stroke mortality.[25]

In 2003, an additional Diet-Heart Hypothesis was described by Siscovick *et al*.[23] Dietary n-3 PUFA increase n-3 PUFA status; higher n-3 PUFA levels favourable affect cardiac ion channel function; the altered channel function modifies the cardiac action potential, which reduces the vulnerability to ventricular fibrillation. Ventricular fibrillation is the major life-threatening arrhythmia that results in sudden cardiac death in the setting of ischemia.[23]

Linoleic acid intake

N-6 PUFA lower LDL-cholesterol, and the ratio of total to HDL-cholesterol.[26] Based on the classic Diet-Heart hypothesis,[23] the positive effects of PUFA on CHD are therefore thought to be mainly mediated by fatty acid induced changes in serum lipid levels. However, in 2010, a meta-analysis of published prospective cohort studies did not demonstrate a protective association with CHD when PUFA were substituted for an iso-caloric amount of saturated fatty acids.[27] In contrast to this finding, another meta-analysis that was published around the same time, showed that the replacement of 5 en% from saturated fat by PUFA was associated with a significantly 13% lower risk for coronary events.[28] In line with this, a meta-analysis of eight randomised trials reported that an increase of PUFA intake of 5 en% would reduce coronary events by 10%.[29] This finding was, however, criticized by other researchers.[30] They stated that the advice to specifically increase

n-6 PUFA, based on mixed n-3/n-6 interventions, was unlikely to provide the intended benefits, and may actually increase the risk of CHD and death.[30]

Alpha-linolenic acid intake

Apart from potential indirect effects of ALA on CVD via conversion into EPA and DHA, it is suggested that ALA could have direct anti-inflammatory,[31,32] anti-arrhythmic,[33] anti-thrombotic,[33,34] or neuroprotective effects.[35] However, others concluded that there is insufficient evidence that ALA influences risk factors for CVD.[36,37] We found no epidemiological studies on the association of ALA intake with stroke. With regard to CHD, the literature is inconclusive. Several prospective cohort studies showed inverse associations of ALA intake with nonfatal myocardial infarction,[38] incident myocardial infarction,[39] incident CHD,[38] fatal CHD,[40-42] sudden cardiac death,[33] or fatal CVD.[41] Other cohort studies suggested no protection of ALA intake against nonfatal myocardial infarction,[33,40] incident CHD,[43] fatal CHD,[33,39,43] sudden death,[38] or fatal CVD.[44] The relation of ALA intake with fatal CHD has been summarized in a meta-analysis of five prospective cohort studies showing that ALA intakes of around 2 g/d were associated with a borderline significant 21% lower risk of fatal CHD (relative risk: 0.79; 95% CI: 0.60-1.04), compared with intakes of 0.8 g/d.[45] In conclusion, the role of ALA in CHD and especially stroke prevention is not clear[31,46,47] and information on the association between ALA intake and stroke is lacking.

Marine n-3 PUFA intake

Numerous prospective epidemiological studies suggest that fish and the fish fatty acids EPA and DHA protect against CVD.[48-50] Already in 1985, Kromhout *et al*. showed that a small amount of fish in the diet was associated with a lower risk of CHD mortality in the Zutphen Study of 852 middle-aged Dutch men.[51] In a meta-analysis of prospective cohort studies, He *et al*. estimated that eating fish once per week was associated with a 15% lower risk of coronary death as compared to a fish intake of less than once per month.[48] The meta-analysis of He *et al*. also showed that the evidence for an inverse association of fish intake and risk of nonfatal myocardial infarction was weak, even though there was a significant inverse association by eating fish 5 times per week or more compared to less than once per month.[48] Several randomized controlled trials (RCT) on fish and fish oil in relation to coronary mortality have been carried out in cardiac patients. The first RCT with fatty fish or fish oil capsules as interventions showed significant reductions in fatal CHD[52,53] and sudden death.[53] Recent meta-analyses of RCT showed that fish oil supplementation significantly reduced fatal CHD[54] and fatal myocardial infarction[55] in coronary patients. Mozaffarian and Rimm[49] combined data from prospective cohort studies and RCT and estimated that a reduction of CHD mortality may be achieved with relatively low intakes of EPA and DHA. Modest

consumption of fish (1-2 servings or ~100-200 g fish per week) was associated with a 36% lower risk of coronary death. They suggested that for the general population an intake of 250 mg/d of EPA-DHA (one serving of fatty fish per week) would be sufficient. Others aim at intakes ~500 mg/d.[56-59]

Several,[60-66] although not all,[67-71] prospective cohort studies showed inverse associations of fish consumption with stroke. In a meta-analysis, He *et al*. summarized prospective cohort studies published through 2003 and concluded that fish consumption once per week compared to less than once per month was related to a 13% (Hazard ratio: 0.87; 95% CI: 0.77-0.98) lower stroke risk.[50] In three cohort studies[62-64] with information on types of stroke, consuming fish more than once a month was associated with a 30-35% lower risk of ischemic stroke, and not with hemorrhagic stroke.[50] Less data are available for EPA-DHA and stroke risk. However, if researchers reported EPA-DHA as well as fish intake in relation to stroke, the results for EPA-DHA were in agreement with the results on fish within these studies.[62,63,67,69,71]

Plasma polyunsaturated fatty acids

Harris *et al*.[72] performed a meta-analysis of 25 (nested) case-control studies and prospective cohort studies on tissue fatty acid composition (18 examining phospholipid-rich and 7 triglyceride-rich samples) and risk of CHD published until 2006. They showed that long-chain n-3 PUFA tissue concentrations, especially DHA, were inversely associated with fatal CHD. However, in this meta-analysis the crude PUFA levels were pooled, i.e. confounders were not taken into account. Furthermore, adipose tissue and various plasma and serum fractions were combined.

There are only a few prospective studies on fatty acid status in relation to incident stroke.[73-75] In a Japanese,[74] but not in an American[73] nested case-control study, total serum linoleic acid and arachidonic acid were inversely associated with incident stroke. ALA in serum cholesteryl esters and phospholipids was inversely associated with stroke risk in the American,[73] but not in the Japanese study.[74] A Swedish nested case-control study found a borderline positive association of EPA-DHA in erythrocytes with ischemic stroke in men but not in women, whereas EPA-DHA status was not associated with total stroke in both sexes.[75] In the Japanese and American studies, EPA and DHA were not related to stroke risk.

The number of studies on PUFA status in relation to cardiovascular diseases is much higher for CHD than for stroke. In epidemiological studies with information on PUFA intake as well as PUFA status, the associations with CHD were stronger for n-3 PUFA status than for intake data.[34,76-78] A disadvantage of the use of biomarkers of PUFA intake is that they do not provide information on absolute PUFA intake, but rather on the contribution of individual PUFA to the total of all fatty acids.

Rationale and outline of the thesis

The objective of this thesis is to assess the relationships of intake and status of the separate n-6 and n-3 PUFA with CHD and stroke. For this purpose, we used data from the "Monitoring Project on Risk Factors for Chronic Diseases" (MORGEN study), which is a Dutch population-based cohort of over 22,000 men and women, aged 20-65 years. Information on diet, lifestyle, and cardiovascular risk factors was collected and blood was drawn at baseline (1993-1997) and participants were followed for fatal and nonfatal cardiovascular disease endpoints. In addition, we used data of the Monitoring Project on Cardiovascular Disease Risk Factors (MP-CVDRF),[79] which is another Dutch population-based cohort with similar data as the MORGEN study, but with longer follow-up for fatal endpoints. In MP-CVDRF, baseline (1987-1991) and follow-up data were collected in ~36,000 subjects aged 20-59 years. **Table 1.1** shows the different topics that are covered in this thesis.

Table 1.1 Outline of the thesis

Fatty acid	Fatty acid	CHD	Stroke
n-6 PUFA	Linoleic acid	Chapter 2 (intake) Chapter 7 (status)	Chapter 8 (status)
	Arachidonic acid	Chapter 7 (status)	Chapter 8 (status)
n-3 PUFA	ALA	Chapter 3 (review) Chapter 4 (intake)	Chapter 4 (intake)
		Chapter 7 (status)	Chapter 8 (status)
	EPA-DHA	Chapter 5 (intake) Chapter 7 (status)	Chapter 6 (intake) Chapter 8 (status)

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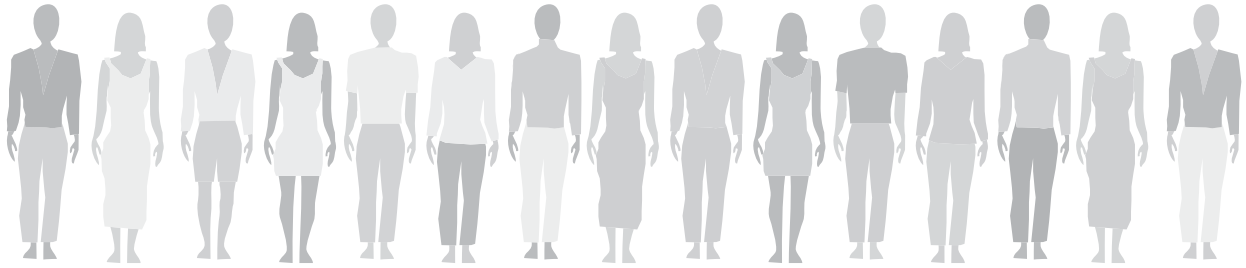

2

Linoleic acid intake, plasma cholesterol, and 10-year incidence of coronary heart disease in 20,000 middle-aged men and women in the Netherlands

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Abstract

We studied the associations of a difference in linoleic acid or carbohydrate intake with plasma cholesterol levels and risk of coronary heart disease (CHD) in a prospective cohort study in the Netherlands. Data on diet (food frequency questionnaire) and plasma total and HDL-cholesterol were available at baseline (1993-1997) of 20,069 men and women, aged 20-65 years, who were initially free of cardiovascular diseases. Incidence of CHD was assessed through linkage with mortality and morbidity registers. During an average of 10 years of follow-up, 280 CHD events occurred. The intake of linoleic acid ranged from 3.6 to 8.0 % of energy (en%) whereas carbohydrate intake ranged from 47.6 to 42.5 en% across quintiles of linoleic acid intake. Linoleic acid intake was inversely associated with total cholesterol and HDL-cholesterol in women but not in men. Linoleic acid intake was not associated with the ratio of total to HDL-cholesterol. No association was observed between linoleic acid intake and CHD incidence with hazard ratios varying between 0.83 and 1.00 (all $P>0.05$) compared to the bottom quintile. We conclude that a 4-5 en% difference in linoleic acid or carbohydrate intake did not translate into either a different ratio of total to HDL-cholesterol or a different CHD incidence.

Introduction

Linoleic acid (C18:2n-6) is an essential polyunsaturated fatty acid (PUFA), and the main fatty acid of the n-6 PUFA family. A linoleic acid intake of 2% of energy (en%) per day is enough to prevent deficiency.[1] Worldwide, the daily intake ranges from 3 en% (India) to 10 en% (Israel).[2,3] In the Netherlands, the average linoleic acid intake is 5-6 en% (~14 g/d).[4-6] N-6 PUFA currently contribute 85-90% of total PUFA intake, largely due to a high consumption of linoleic acid-rich vegetable oils.[7]

In 2009, the American Heart Association summarised the scientific evidence on the association between n-6 PUFA and cardiovascular diseases (CVD) and advised to consume 5-10 en% of n-6 PUFA per day.[8] N-6 PUFA have a favourable effect on LDL-cholesterol and the ratio of total to HDL-cholesterol.[9] The positive effects of PUFA on CHD are therefore thought to be mainly mediated by fatty acid-induced changes in serum lipid levels. The total to HDL-cholesterol ratio is improved by substituting PUFA for saturated fatty acids, but also by substituting PUFA for carbohydrates.[10] Furthermore, carbohydrates also increase triglycerides, which are an independent risk factor for CHD.[11]

A recent meta-analysis of published prospective cohort studies did not demonstrate a positive effect of a higher PUFA intake in exchange with an isocaloric amount of saturated fatty acids on CHD.[12] However, another meta-analysis based on individual data that specifically addressed the isocaloric exchange of saturated fatty acids and PUFA showed that the replacement of 5% of energy from saturated fat by PUFA was significantly associated with a 13% lower risk for coronary events, whereas the replacement of saturated fat by carbohydrates showed a significant direct association with CHD.[13] It has been suggested that replacement of saturated fat by carbohydrates, mainly refined carbohydrates, may exacerbate atherogenic dyslipidemia.[14]

The aim of the present study was to evaluate the intake of linoleic acid, as an isocaloric substitute for carbohydrates, in relation to both plasma cholesterol levels and 10-year incidence of CHD in a population-based cohort of over 20,000 adults in the Netherlands.

Methods

Design and study population

The 'Monitoring Project on Risk Factors for Chronic Diseases' (MORGEN) study is a Dutch population-based cohort of 22,654 men and women, aged 20-65 years. MORGEN is part of the European Prospective Investigation into Cancer and Nutrition study.[15] Baseline (1993-1997) information on diet, plasma cholesterol levels, lifestyle, and cardiovascular risk factors was collected and participants were followed up for cardiovascular disease end points. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki

and all procedures involving human subjects/patients were approved by the Medical Ethics Committee of TNO Prevention and Health (Leiden, The Netherlands). Written informed consent was obtained from all subjects.

For the present study, participants who did not provide informed consent for vital status follow-up (n=701) were excluded, as well as 72 participants without dietary information and 97 participants with extreme energy intakes (<2,094 or >18,844 kJ for women and <3,350 or > 20,938 kJ for men). Furthermore, participants with a history of myocardial infarction (MI) or stroke at baseline were excluded (n=442). We also excluded participants who reported the use of serum lipid- modifying agents (n=203) or antihypertensive drugs (n=887), and 180 participants with diabetes resulting in 20,069 participants (8,988 men and 11,081 women).

Dietary assessment

The habitual diet was assessed with a validated self-administered 178-item food-frequency questionnaire (FFQ) covering the previous year.[16,17] The FFQ included foods that covered the intake of foods and nutrients relevant to chronic disease aetiology for at least 90% of the national mean intake. Participants indicated the consumption of main food groups in times per day, per week, per month, per year or as never, combined with questions on the relative intakes of foods within food groups (seldom/never, sometimes, often, mostly/always). Nutrient intakes were calculated with the Dutch food composition table of 1998. For individual fatty acids, we used the table of 2001, because the values were more complete. Total energy intake was calculated as the sum of energy from fat, carbohydrates, and protein.

The reproducibility (estimated by two repeated measurements) and the relative validity (intake assessed by the FFQ compared to intakes assessed by 12 monthly 24-h recalls) of the FFQ for food groups and some nutrients were assessed among 121 Dutch men and women.[16,17] The Spearman rank correlations for the reproducibility of the FFQ after 6 months for total energy intake were 0.90 for men and 0.80 for women. Rank correlations were 0.83 and 0.77 for fat, 0.86 and 0.75 for protein, and 0.91 and 0.85 for carbohydrates, in men and women, respectively. The relative validity of the FFQ for total energy intake was 0.77 for men and 0.62 for women. Rank correlations were 0.74 and 0.63 for fat, 0.68 and 0.56 for protein, and 0.75 and 0.69 for carbohydrates, in men and women respectively.

Plasma lipid measurements

Total cholesterol and high-density lipoprotein (HDL) cholesterol were measured in nonfasting EDTA plasma at the Lipid Reference Laboratory of the Erasmus Medical Center Rotterdam, using enzymatic methods. Total cholesterol was measured using an enzymatic method; HDL-cholesterol was determined in the supernatant after precipitation of apoB-containing lipoproteins with phosphotungstic acid/$MgCl_2$. Performance for enzymatic total and HDL-cholesterol

measurements fulfilled the National Cholesterol Education Program recommendations throughout the study period.[18]

Mortality and morbidity

Vital status was checked through linkage with the national population register. Participants were followed for the occurrence of CHD by linkage with Statistics Netherlands for cause-specific mortality. Information on nonfatal events was provided by the national hospital discharge register based on a validated probabilistic linkage method described in more detail elsewhere.[19] It has been shown that on the national level, data from the Dutch hospital discharge register can be uniquely matched to a single person for at least 88% of the hospital admissions.[19] Incident CHD included fatal CHD (I20-I25), fatal and nonfatal cardiac arrest (I46), and non-fatal MI (I21-I22) according to the International Classification of Diseases (ICD-10, WHO). For hospital admissions and for causes of death coded until 1 January, 1996, corresponding ICD9 codes were used. Participants were followed up until death, incident CHD, date of loss-to-follow-up due to emigration out of the Netherlands (n=693) or 1 January 2006, whichever came first.

Other baseline characteristics

Body weight, height and blood pressure were measured by trained research nurses. Self-administered questionnaires were used to assess the presence of diabetes, MI and stroke at baseline, medication use, parental history of MI, educational level and cigarette smoking.[20] Alcohol intake (based on the FFQ) was calculated in glasses/d. Baseline physical activity was assessed with a validated questionnaire in 76% of the cohort who were enrolled between 1994 and 1997.[21] For this subset, we calculated whether participants were engaged in activities with a metabolic equivalent score ≥ 4 (yes/no). Cycling (yes/no) and sports (yes/no) were previously shown to be significantly inversely related to CVD incidence in this population.[22]

Statistical analysis

Participants' characteristics by quintiles of linoleic acid intake expressed as en% are presented as mean values and standard deviation, medians with interquartile ranges or percentages. Correlations between the energy-adjusted intakes of different types of fatty acids were assessed with the Spearman rank correlation test.

Mean plasma levels of total and HDL-cholesterol and the ratio of total to HDL-cholesterol by quintiles of linoleic acid intake (en%) were computed using general linear models. *P* for trend of plasma cholesterol levels were calculated based on the continuous distribution of linoleic acid intake. We used Cox proportional hazards models to estimate relative risks for the incidence of CHD across quintiles of linoleic acid intake at baseline. Hazard ratios (HR) with

95% confidence intervals (CI) were obtained using the bottom quintile of linoleic acid intake as the reference category. The proportional-hazards assumption was tested and not rejected based on Schoenfeld residuals and visual inspection. In model 1, we adjusted for total energy intake (kJ/d), age and sex. In model 2, we additionally adjusted for body mass index (kg/m^2), alcohol intake (glasses/d), current cigarette smoking, high educational level (completed higher vocational training or university) (yes/no), parental history of premature CHD (MI of father before the age of 55 years or MI of mother before the age of 65 years) (yes/no). In model 3, we added intakes of fibre (g/d), protein, saturated fatty acids, *cis*-monounsaturated fat, *trans*-fat and PUFA other than linoleic acid (all in en%). The estimated HR of the full model can be interpreted as an isocaloric replacement of carbohydrates with linoleic acid.

We assessed the impact of adjustments for systolic blood pressure, the total to HDL-cholesterol ratio and physical activity. Effect modification was evaluated for age and sex by adding product terms to the models. All statistical analyses were performed with SAS (version 9.1; SAS Institute, Inc., Cary, NC, USA). Two-sided *P*-values <0.05 were considered statistically significant.

Results

Population characteristics

Participants were on average 41.5 (SD 11.1) years at baseline, and 45% was male. The average intake of linoleic acid was 13.9 (SD 5.9) g/d or 5.6 (SD 1.6) en%. Linoleic acid comprised 79% of total PUFA intake. During 8-13 years of follow-up (median 10.5 y), 199 men and 81 women experienced a CHD event, of which 19% was a fatal event.

The main sources of linoleic acid intake were margarines (21%), oils (13%), bread (12%), nuts (10%), pork meat (10%), and sauces (9%). Mean linoleic acid intake more than doubled across quintiles (Q), from 3.6 en% in Q1 to 8.0 en% in Q5. Saturated and *trans*-fatty acids did not differ across quintiles and carbohydrate intake decreased from 47.6 to 42.5 en%. Polysaccharides did not differ between quintiles, whereas mono- and disaccharides decreased from 25 to 19 en% (**Table 2.1**). The Spearman correlations with linoleic acid were 0.98 for total PUFA, 0.43 for total fat, 0.32 for *cis*-MUFA, and -0.35 for mono and disaccharides.

Linoleic acid, plasma lipid levels and CHD

We observed interaction of gender on the association between linoleic acid intake and cholesterol levels. *P*-values for interaction were <0.0001 for total cholesterol, 0.01 for HDL-cholesterol, and 0.37 for the ratio of total to HDL-cholesterol. In women, linoleic acid intakes were inversely associated with plasma total cholesterol levels in the fully adjusted model, with a mean total cholesterol level of 5.28 mmol/l (203 mg/dl) in Q1 and 5.14 mmol/l (198 mg/dl)

Table 2.1 Baseline characteristics of 20,069 Dutch men and women, aged 20-65 years, by quintiles of energy percentages of linoleic acid intake[a] (mean values and standard deviations, unless indicated)

	Q1	Q2	Q3	Q4	Q5
n	4,013	4,014	4,014	4,014	4,014
Male sex, %	45	42	43	46	48
Age, y	41.6 ± 11.8	40.5 ± 11.2	40.7 ± 10.9	41.2 ± 10.7	43.3 ± 10.6
PUFA					
g/d	11.9 ± 4.0	15.0 ± 4.4	17.0 ± 5.0	19.6 ± 5.8	23.9 ± 7.6
en%	4.9 ± 0.6	6.1 ± 0.4	6.9 ± 0.4	7.8 ± 0.5	9.6 ± 1.2
Linoleic acid					
g/d	8.8 ± 3.0	11.4 ± 3.4	13.3 ± 3.9	15.8 ± 4.7	20.0 ± 6.6
en%	3.6 ± 0.5	4.6 ± 0.2	5.4 ± 0.2	6.3 ± 0.3	8.0 ± 1.1
α-linolenic acid					
g/d	1.1 ± 0.4	1.3 ± 0.5	1.4 ± 0.5	1.6 ± 0.6	1.7 ± 0.7
en%	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.7 ± 0.2
Cis-MUFA					
g/d	27.8 ± 10.5	30.3 ± 10.5	31.2 ± 10.8	32.7 ± 11.4	33.7 ± 12.4
en%	11.4 ± 2.1	12.3 ± 2.0	12.5 ± 2.1	12.9 ± 2.1	13.5 ± 2.3
TFA					
g/d	3.7 ± 1.9	3.9 ± 1.9	3.9 ± 1.8	3.9 ± 1.9	3.7 ± 1.8
en%	1.5 ± 0.5	1.6 ± 0.5	1.6 ± 0.5	1.5 ± 0.5	1.4 ± 0.5
SFA					
g/d	36.9 ± 14.5	37.5 ± 13.3	37.2 ± 13.1	38.0 ± 13.3	36.8 ± 13.2
en%	15.1 ± 3.0	15.1 ± 2.5	14.9 ± 2.4	15.0 ± 2.3	14.7 ± 2.3
P/S ratio	0.32	0.40	0.46	0.52	0.65
Cholesterol, mg/d	244.0 ± 96.8	245.8 ± 92.1	241.8 ± 89.9	244.2 ± 90.2	231.3 ± 91.0
Total fat					
g/d	81.2 ± 29.7	87.4 ± 29.0	89.9 ± 29.6	95.0 ± 31.4	98.7 ± 33.3
en%	33.3 ± 5.2	35.3 ± 4.5	36.1 ± 4.5	37.6 ± 4.4	39.4 ± 4.5
Total carbohydrate, en%	47.6 ± 5.5	46.0 ± 4.8	45.3 ± 4.8	44.1 ± 4.7	42.5 ± 4.8
Mono and disaccharides, en%	25.0 ± 6.2	22.5 ± 5.3	21.6 ± 5.2	20.5 ± 5.1	19.1 ± 4.9
Polysaccharides, en%	22.6 ± 4.7	23.4 ± 4.3	23.6 ± 4.2	23.6 ± 4.3	23.4 ± 4.2
Fibre, g/d	23.8 ± 7.4	24.4 ± 7.0	24.8 ± 7.0	25.3 ± 7.2	25.6 ± 7.3
Protein, en%	16.1 ± 2.7	15.8 ± 2.3	15.7 ± 2.2	15.5 ± 2.1	15.3 ± 2.1
Energy intake,[a] MJ/d	9.0 ± 2.8	9.1 ± 2.7	9.2 ± 2.7	9.3 ± 2.7	9.2 ± 2.7
Body mass index, kg/m^2	24.9 ± 3.8	24.9 ± 3.9	24.9 ± 3.8	24.8 ± 3.7	24.9 ± 3.9
Body weight, kg	74.2 ± 13.6	74.1 ± 13.3	74.4 ± 13.2	74.3 ± 13.1	74.5 ± 13.8
Current smoking, %	35	36	36	37	39
Alcohol consumption,[b] glasses/d	0.4 (0.1-1.7)	0.6 (0.1-1.7)	0.7 (0.1-1.9)	0.7 (0.1-2.0)	0.8(0.1-2.0)
Highly educated,[c] %	20	23	27	27	27
Physically active,[d] %					
Engaged in:					
Sports	38	38	39	38	34
Cycling	57	60	61	62	58
Parental history of MI, %	8	8	9	10	9
SBP, mm Hg	120.4 ± 15.5	119.8 ± 15.5	119.3 ± 15.3	119.3 ± 15.0	120.7 ± 16.3
DBP, mm Hg	76.4 ± 10.3	76.0 ± 10.4	75.8 ± 10.2	75.8 ± 10.2	76.4 ± 10.7
Plasma total cholesterol, mmol/l	5.3 ± 1.0	5.3 ± 1.0	5.2 ± 1.0	5.3 ± 1.0	5.3 ± 1.1
Plasma HDL-cholesterol, mmol/l	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4
Total cholesterol / HDL-cholesterol	4.1 ± 1.5	4.1 ± 1.4	4.1 ± 1.5	4.1 ± 1.5	4.2 ± 1.5

Abbreviations: Q, quintile; en%, % of energy; PUFA, polyunsaturated fatty acids; *cis*-MUFA, *cis*-monounsaturated fatty acids; TFA, *trans*-fatty acids; SFA, saturated fatty acids; MI, myocardial infarction; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein; P/S ratio, ratio of PUFA/SFA.

[a] Total energy excluding alcohol intake.

[b] Median with interquartile range.

[c] University or higher vocation training.

[d] Available for participants enrolled between 1994 and 1997 (n=15,423).

in Q5 (*P*-trend <0.0001). In men, however, linoleic acid intake was not associated with total cholesterol. Linoleic acid intakes were inversely associated with plasma HDL-cholesterol across quintiles both in men and women, although this inverse association did not reach statistical significance in men.

Linoleic acid intake was inversely, but not significantly, associated with the ratio of total to HDL-cholesterol in women, with ratio values between 3.65 (Q1) and 3.59 (Q5), whereas a positive, yet also non-significant, association was observed in men, with ratios varying between 4.65 (Q1) and 4.73 (Q5; **Table 2.2**).

After adjustment for potential confounders, linoleic acid intake was not associated with incident CHD. HR varied between 0.83 and 1.00 (all *P*>0.05) compared with the bottom quintile of linoleic acid intake (**Table 2.3**). The HR of incident CHD for a 5% higher energy intake of linoleic acid with a concurrent lower intake of carbohydrates was 1.01 (95% CI: 0.86-1.19).

We observed no interaction of sex or age (≤50 or >50 years) and linoleic acid intake in relation with incident CHD (data not shown). HR (95% CI) for incident CHD after additional inclusion of the ratio of total to HDL-cholesterol and systolic blood pressure in the multivariable models were 0.92 (95% CI: 0.62-1.36), 0.82 (95% CI: 0.55-1.24), 1.00 (95% CI: 0.68-1.48), 0.86 (95% CI: 0.57-1.30) for Q2-Q5 compared with Q1, respectively. For the subgroup with information on physical activity (n=15,423), the full model with and without physical activity yielded similar results (data not shown). Models based on isocaloric substitution with saturated fatty acids did not differ from the models based on substitution with carbohydrates (data not shown).

Discussion

In this large Dutch population-based cohort, a higher linoleic acid and concurrent lower carbohydrate intake was inversely associated with total cholesterol and HDL-cholesterol in women, but not in men. Linoleic acid intake was neither related to the ratio of total to HDL-cholesterol nor to CHD incidence.

In the present population-based study, the intakes of saturated fat and *trans*-fat were similar across the quintiles of linoleic acid intake and only carbohydrates varied between high and low linoleic acid intake. Our data set therefore allowed the analysis of real differences in intake between participants instead of statistically modelling these differences. Another strength of the present study was the almost complete follow up of mortality. Furthermore, the procedure of identification of non-fatal events was validated in 36% of the participants of the present study by comparison against the clinical registry of the Cardiology Department of the Maastricht University Hospital. This showed a relatively high sensitivity (84%) and positive predictive value (97%) for MI.[23] In addition, we also had detailed information on many potential confounders.

Table 2.2 Adjusted cholesterol levels by quintiles of energy percentages of linoleic acid intake in 20,069 Dutch men and women[a,b] (mean values with their standard errors)

			Model 1[c]	Model 2[d]	Model 3[e]
	n	Median intake, en%	Mean ± SE	Mean ± SE	Mean ± SE
Men			Total cholesterol, mmol/l		
Q1	1,797	3.7	5.27 ± 0.02	5.26 ± 0.02	5.25 ± 0.02
Q2	1,798	4.7	5.30 ± 0.02	5.30 ± 0.02	5.30 ± 0.02
Q3	1,798	5.4	5.26 ± 0.02	5.26 ± 0.02	5.26 ± 0.02
Q4	1,798	6.3	5.33 ± 0.02	5.34 ± 0.02	5.35 ± 0.02
Q5	1,797	7.8	5.27 ± 0.02	5.28 ± 0.02	5.29 ± 0.02
P-trend			*0.90*	*0.49*	*0.31*
			HDL-cholesterol, mmol/l		
Q1	1,797	3.7	1.19 ± 0.01	1.20 ± 0.01	1.21 ± 0.01
Q2	1,798	4.7	1.20 ± 0.01	1.20 ± 0.01	1.20 ± 0.01
Q3	1,798	5.4	1.18 ± 0.01	1.18 ± 0.01	1.18 ± 0.01
Q4	1,798	6.3	1.19 ± 0.01	1.19 ± 0.01	1.18 ± 0.01
Q5	1,797	7.8	1.20 ± 0.01	1.20 ± 0.01	1.19 ± 0.01
P-trend			*0.38*	*0.61*	*0.16*
			Total cholesterol/HDL-cholesterol		
Q1	1,797	3.7	4.72 ± 0.04	4.70 ± 0.03	4.65 ± 0.04
Q2	1,798	4.7	4.68 ± 0.04	4.68 ± 0.03	4.67 ± 0.03
Q3	1,798	5.4	4.73 ± 0.04	4.72 ± 0.03	4.73 ± 0.03
Q4	1,798	6.3	4.74 ± 0.04	4.77 ± 0.03	4.79 ± 0.03
Q5	1,797	7.8	4.69 ± 0.04	4.70 ± 0.03	4.73 ± 0.04
P-trend			*0.68*	*0.78*	*0.06*
Women			Total cholesterol, mmol/l		
Q1	2,216	3.8	5.28 ± 0.02	5.28 ± 0.02	5.28 ± 0.02
Q2	2,216	4.6	5.29 ± 0.02	5.28 ± 0.02	5.28 ± 0.02
Q3	2,217	5.3	5.21 ± 0.02	5.21 ± 0.02	5.21 ± 0.02
Q4	2,216	6.2	5.22 ± 0.02	5.22 ± 0.02	5.22 ± 0.02
Q5	2,216	7.7	5.13 ± 0.02	5.14 ± 0.02	5.14 ± 0.02
P-trend			*<0.0001*	*<0.0001*	*<0.0001*
			HDL-cholesterol, mmol/l		
Q1	2,216	3.8	1.52 ± 0.01	1.52 ± 0.01	1.53 ± 0.01
Q2	2,216	4.6	1.52 ± 0.01	1.53 ± 0.01	1.53 ± 0.01
Q3	2,217	5.3	1.52 ± 0.01	1.52 ± 0.01	1.52 ± 0.01
Q4	2,216	6.2	1.51 ± 0.01	1.51 ± 0.01	1.51 ± 0.01
Q5	2,216	7.7	1.52 ± 0.01	1.52 ± 0.01	1.51 ± 0.01
P-trend			*0.89*	*0.25*	*0.002*
			Total cholesterol/HDL-cholesterol		
Q1	2,216	3.8	3.67 ± 0.02	3.66 ± 0.02	3.65 ± 0.02
Q2	2,216	4.6	3.66 ± 0.02	3.65 ± 0.02	3.64 ± 0.02
Q3	2,217	5.3	3.62 ± 0.02	3.62 ± 0.02	3.62 ± 0.02
Q4	2,216	6.2	3.65 ± 0.02	3.65 ± 0.02	3.66 ± 0.02
Q5	2,216	7.7	3.57 ± 0.02	3.58 ± 0.02	3.59 ± 0.02
P-trend			*0.001*	*0.008*	*0.10*

Abbreviations: en%, % of energy; Q, quintile; HDL-cholesterol, high density lipoprotein cholesterol.

[a] Total cholesterol was missing for n=113 and HDL-cholesterol was missing for n=123.

[b] Stratified analysis was based on sex-specific quintiles of linoleic acid.

[c] Means adjusted for age, sex, and total energy intake (for total cholesterol: n=8,951 in men and n=11,005 in women for HDL-cholesterol and the ratio of total to HDL-cholesterol: n=8,948 in men and n=10,998 in women).

[d] Means additionally adjusted for smoking, body mass index, educational level, parental history of myocardial infarction, and alcohol intake (for total cholesterol: n=8,876 in men and n=10,909 in women for HDL-cholesterol and the ratio of total to HDL-cholesterol: n=8,875 in men and n=10,902 in women).

[e] Means additionally adjusted for intake of dietary fibre, protein, saturated fatty acids, monounsaturated fatty acids, *trans*-fatty acids, polyunsaturated fatty acids other than linoleic acid (for total cholesterol: n=8,876 in men and n=10,909 in women for HDL-cholesterol and the ratio of total to HDL-cholesterol: n=8,875 in men and n=10,902 in women).

Table 2.3 Associations of linoleic acid intake with incident CHD in 20,069 Dutch men and women (numbers, hazard ratios and 95% confidence intervals)

			Model 1[a]		Model 2[b]		Model 3[c]	
	Median intake	Cases n	HR	95% CI	HR	95% CI	HR	95% CI
Q1 (n=4,013)	3.7	61	1.0 (ref)		1.0 (ref)		1.0 (ref)	
Q2 (n=4,014)	4.7	50	0.95	(0.65-1.38)	0.93	(0.63-1.35)	0.90	(0.61-1.33)
Q3 (n=4,014)	5.4	46	0.86	(0.59-1.26)	0.86	(0.58-1.27)	0.83	(0.56-1.24)
Q4 (n=4,014)	6.2	61	1.03	(0.72-1.47)	1.04	(0.73-1.50)	1.00	(0.68-1.47)
Q5 (n=4,014)	7.7	62	0.91	(0.64-1.29)	0.94	(0.65-1.34)	0.90	(0.60-1.36)
Per 2%			1.00	(0.87-1.15)	1.02	(0.89-1.17)	1.01	(0.86-1.19)

Abbreviations: CHD, coronary heart disease; HR, hazard ratio.

Values are hazard ratios with 95% confidence intervals in quintiles (Q1-Q5) of linoleic acid intake, using Q1 as the reference category.

[a] Adjusted for age, sex, and total energy intake (n=20,069).

[b] Additionally adjusted for smoking, body mass index, educational level, parental history of myocardial infarction, and alcohol intake (n=19,896).

[c] Additionally adjusted for dietary fibre, protein, saturated fatty acids, *cis*-monounsaturated fatty acids, *trans*-fatty acids, and PUFA other than linoleic acid (n=19,896).

The present study also had limitations. Misclassification of subjects for linoleic acid intake may have occurred. However, we excluded participants with a history of MI or stroke, and also participants who used cholesterol lowering or blood pressure lowering medication, because these may have changed their diet. We therefore consider potential misclassification at baseline to be random rather than dependent on disease outcome. Multiple simultaneous and partly opposite effects of diet and lifestyle on CHD incidence had to be taken into account, including correlated types of fatty acids, partly due to presence in the same foods. Like in any other epidemiological study on diet and CHD, this may also have affected results in the present study. Although we attempted to disentangle the various possible simultaneous effects with the present statistical models, it is impossible to completely rule out confounding.

In the present study, linoleic acid intake was not significantly associated with the plasma total to HDL-cholesterol ratio. A meta-analysis of controlled dietary intervention studies showed that the replacement of 1 en% of carbohydrates by PUFA would result in a reduction of the ratio of total to HDL-cholesterol by 0.032. In observational studies of adults between 40 and 59 years, each 1 unit lower total to HDL-cholesterol ratio was associated with a 44% lower risk of CHD.[9] In the population of the present study, derived from controlled dietary intervention studies, the predicted difference of the total to HDL ratio between highest and lowest quintiles of linoleic acid intake was -0.15,[10] which would correspond to an approximately 7% lower CHD incidence. However, such a modest difference is difficult to detect considering the errors in observational food intake data.

A linoleic acid intake ranging between 3.6-8.0 en% was not significantly associated with incident CHD in the present study. These results are in line with those from the cohort studies in Finnish men,[24] Danish men and women,[25] and American men,[26] which also used models of isocaloric substitution of PUFA for carbohydrates. A similar model was used in the Nurses' Health Study. However, in that study with 80,000 women (1,766 events) PUFA intake, ranging from 4.1-7.4 en%, was inversely associated with a 25% (95% CI: 8-40%) lower CHD incidence in Q5 compared to Q1.[27] Observational studies in the USA or Western Europe mostly covered relatively small ranges of intake of 5 to 10 en%. To find associations within this range will be complicated by measurement error of intake in single dietary assessments.[28] Therefore, in observational studies differences in linoleic acid intake may not translate into the predicted, although modest, differences in cholesterol and CHD risk.

On the basis of eight randomised trials, it has been shown that an increase in PUFA intake of 5% of energy was significantly associated with a 10% lower risk of coronary events.[29] However, this effect size was estimated from large contrasts of PUFA intake (on average 5 v. 15 en%) between the intervention and control groups. Additionally, the PUFA interventions were mostly a combination of both a higher intake of n-6 and n-3 PUFA whereas in the present study, we adjusted for PUFA other than linoleic acid. A recent meta-analysis separated the trials in the meta-analysis of Mozaffarian *et al*.[29] into those on n-6 PUFA only and those with combined n-6 and n-3 PUFA interventions. The authors concluded[30] that there was no indication of benefit of n-6 PUFA, although this statement was based on only 2 trials. However, it is clear from the information presented in that paper that the effect of a 5 en% difference in n-6 PUFA intake is less than the 10% difference in CHD incidence calculated from the dietary interventions for total PUFA.

In conclusion, in this large population-based study in the Netherlands, a 4-5 en% difference in linoleic acid or carbohydrate intake did not translate into either a different ratio of plasma total to HDL-cholesterol or a different CHD incidence.

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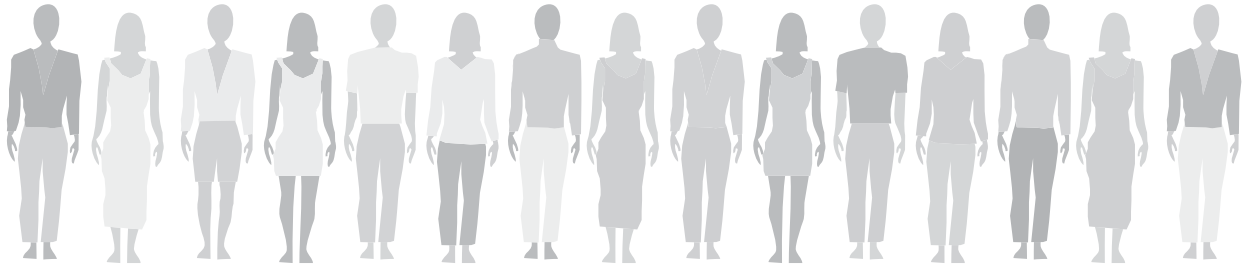

3

Alpha-linolenic acid: Is it essential to cardiovascular health?

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Abstract

There is a large body of scientific evidence that has been confirmed in randomized controlled trials indicating a cardioprotective effect for omega-3 fatty acids from fish. For alpha-linolenic acid (ALA), which is the omega-3 fatty acid from plants, the relation to cardiovascular health is less clear. We reviewed the recent literature on dietary ALA intake, ALA tissue concentrations, and cardiovascular health in humans. Short-term trials (6-12 weeks) in generally healthy participants mostly showed no or inconsistent effects of ALA intake (1.2-3.6 g/d) on blood lipids, low-density lipoprotein oxidation, lipoprotein(a), and apolipoproteins A-I and B. Studies of ALA in relation to inflammatory markers and glucose metabolism yielded conflicting results. With regard to clinical cardiovascular outcomes, there is observational evidence for a protective effect against nonfatal myocardial infarction. However, no protective associations were observed between ALA status and risk of heart failure, atrial fibrillation, and sudden death. Findings from long-term trials of ALA supplementation are awaited to answer the question whether food-based or higher doses of ALA could be important for cardiovascular health in cardiac patients and the general population.

Introduction

Omega-3 (n-3) fatty acids can be divided into alpha-linolenic acid (ALA; C18:3n-3) from plant origin, and eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) from seafood. Because the human body lacks the enzymatic capacity to synthesize ALA, it is essential to obtain it from the diet.

The estimated average ALA intake in the United States and most European countries is 1.3 to 1.7 g/d.[1-3] The Institute of Medicine (IOM) of the National Academies established Dietary Reference Intakes for macronutrients in 2002.[4] For ALA, the Adequate Intake (AI) was set at 1.6 g/d for men and 1.1 g/d for women.[4] The IOM noted that intakes of n-3 fatty acids above the AI may confer additional health benefits, especially with respect to cardiovascular health. Many advisory boards consider ALA intakes greater than 1.5 g/d important for human health.[1] To achieve an adequate ALA intake, food sources such as flaxseed and flaxseed oil, walnuts and walnut oil, and canola and soybean oil are recommended.

For EPA and DHA from fish, there is a vast amount of epidemiologic evidence indicating a cardioprotective effect. This evidence has been confirmed in randomized controlled trials.[5-8] For ALA, the relation with cardiovascular health is less clear. Several large, prospective cohort studies in the United States have shown inverse associations of ALA intake with risk of cardiovascular diseases, but other epidemiologic studies have been inconclusive.[9-11] A meta-analysis of observational studies showed that increased intake of ALA might reduce coronary heart disease (CHD) mortality by 21%, although this was not statistically significant.[11] In the Lyon Diet Heart Study, a randomized controlled trial in coronary patients, consumption of a Mediterranean-type diet that included an additional daily intake of roughly 1 g of ALA significantly decreased the risk of cardiac death and nonfatal myocardial infarction (MI) by more than 60%.[12] This study, however, was not specifically designed to assess the effect of ALA supplementation, and many dietary factors differed between the experimental and control group. Since then, no randomized controlled trials of ALA and cardiovascular events have been published. This article summarizes the current literature (published after 2008) on dietary ALA intake, ALA tissue levels, and cardiovascular health in humans.

Effect of ALA supplementation on cardiovascular risk factors

Several trials have recently been published on the effect of ALA supplementation on blood lipids, inflammatory markers, and other indicators of cardiovascular health (**Table 3.1**).[13,14•,15-17,18••] Trials were mostly of relatively short duration (6-12 weeks) and ALA doses ranged from 1.2 to 3.6 g/d. There was one long-term trial (52 weeks) in which a high ALA dose of 8.8 g/d was given.[18••] Increased ALA intake was achieved by means of flaxseed oil,[13,15] ALA-enriched margarine,[14•] or other ALA-enriched foods.[16,17,18••]

Kaul et al.[13] studied the effects of low-dose supplementation (2 g/d, by means of capsules) of flaxseed oil, fish oil, and hempseed oil in 86 healthy Canadian men and women about 34 years

Table 3.1 Overview of randomized controlled trials of increased alpha-linolenic acid intake and cardiovascular risk factors published between January 2008 and June 2010

Study	Year	Population	Design	Outcome for ALA
Kaul et al.[13]	2008	88 healthy non-smoking Canadian men and premenopausal women, aged 33 y	12-week, double-blind, parallel randomized controlled trial; sunflower oil (placebo), flaxseed oil (~1 g/d ALA), hempseed oil (0.3 g/d ALA), or fish oil (0.6 g/d EPA+DHA)	Plasma ALA levels increased ($P<0.05$) after 6 wk; no differences in total cholesterol, LDL-C, HDL-C, TG, LDL oxidation, platelet aggregation, or inflammation markers (CRP, TNF-α)
Egert et al.[14•]	2009	79 healthy non-smoking German men and premenopausal women, aged 19-45 y	6-week, double-blind, parallel randomized controlled trial; ALA (3.4 g/d), EPA (2.2 g/d), or DHA (2.3 g/d) via enriched margarines	LDL-ALA levels increased ($P<0.05$); fasting serum TG decreased ($P<0.05$); no differences in total cholesterol, LDL-C, or HDL-C
Barceló-Coblijn et al.[15]	2008	62 American men >40 y of age	12-week, parallel randomized controlled trial; different doses of flax oil, fish oil, and sunflower oil in capsules; ALA doses were 1.2 g/d, 2.4 g/d, and 3.6 g/d	2.4 and 3.6 g/d of ALA significantly increased erythrocyte ALA and EPA levels; no differences in inflammation markers (CRP, TNF-α, sVCAM-1), total cholesterol, TG, or HDL-C
Sioen et al.[16]	2009	59 healthy Belgian male prisoners	12-week single-blind study; diet with 3.2 g/d extra ALA	No effect on waist circumference, weight, BMI, systolic blood pressure; diastolic blood pressure decreased and HDL-C increased in non-smokers
Bloedon et al.[17]	2008	62 men and post-menopausal women from Philadelphia, aged 44-75 y, with hypercholesterolemia	10-week, blind, parallel randomized controlled trial with low-fat diet with extra flaxseed or with wheat bran (control); ALA dose of 3.4 g/d	Serum ALA levels increased; LDL-C was decreased after 5 wk but not after 10 wk; lipoprotein(a) and improved insulin sensitivity (HOMA index); no effect on inflammation (IL-6, hs-CRP) or oxidative stress (ox-LDL, urinary isoprostane); HDL-C was reduced
Dodin et al.[18••]	2008	199 Canadian menopausal women, aged 49-65 y	52-week, blind parallel trial; 40 g/d flaxseed or wheat germ; ALA dose of 8.8 g/d	Serum ALA levels increased; modest effects on apolipoproteins A-I and B; no effects on LDL electrophoretic characteristics or markers of hemostasis and inflammation

Abbreviations: ALA, alpha-linolenic acid; BMI, body mass index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDL-C, high-density lipoprotein cholesterol; HOMA, homeostasis model assessment; hs-CRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; LDL-C, low-density lipoprotein cholesterol; sVCAM-1, soluble vascular cell adhesion molecule-1; TG, triglycerides; TNF, tumor necrosis factor.

of age in a 12-week, randomized, double-blind, placebo-controlled trial. Flaxseed oil (ALA dose ~1 g/d) increased plasma ALA by 30% to 40%, had a small nonsignificant effect on plasma EPA (7%-8%), and no effect on plasma DHA. None of the treatments affected serum total, low-density lipoprotein (LDL), or high-density lipoprotein (HDL) cholesterol, triglycerides, LDL oxidation, platelet aggregation, or inflammatory markers.[13] In another randomized trial in 62 healthy men approximately 40 years of age in the United States, participants were randomized to flax oil (ALA doses of 1.2, 2.4, or 3.6 g/d), fish oil (0.6 or 1.2 g/d), or sunflower oil for 12 weeks.[15] Increasing doses of flax oil caused elevation of the ALA content in erythrocyte membranes by 40% to 80%, and also of EPA (20%-35%), but not of DHA. None of the treatments altered plasma inflammatory markers (C-reactive protein [CRP], tumor necrosis factor [TNF]-α, soluble vascular cell adhesion molecule-1 [sVCAM-1]), plasma total or HDL cholesterol, or triglycerides.[15]

Bloedon et al.[17] enrolled 62 American men and post-menopausal women aged 44 to 75 years with hypercholesterolemia. All participants were on a low-fat, low-cholesterol diet and subsequently randomized to flax-based or wheat-based products for 10 weeks. ALA intake in the flaxseed group was 3.4 g/d higher than in the control group. Flax-based products improved insulin sensitivity and reduced plasma LDL cholesterol by 7% to 13% and lipoprotein(a) by 14% compared with wheat-based products. In men, there was also a decrease in HDL cholesterol. Treatment had no effect on markers of inflammation (interleukin-6, high-sensitivity CRP) or oxidative stress (oxidized LDL, urinary isoprostanes).[17] The extent to which the observed effects of the flax-based diet were due to its ALA content or other components of flax seeds is not clear.

In a single-blind, 12-week field study among 59 healthy male prisoners (mean age of 42 years) in Belgium, daily ALA intake was increased from 2.8 to 4.9 g/d by means of ALA-enriched foods without changing the linoleic acid content of the diet.[16] All individuals were first on a regular diet, which was followed by an ALA-rich diet, and no randomization was applied. EPA and DHA, but not ALA, were assessed in platelet phospholipids, which showed no significant changes during ALA intervention. Body weight, waist circumference, and systolic blood pressure did not change, and there were no effects of ALA on plasma total and LDL cholesterol, triglycerides, apolipoproteins A-I and B, glucose, and CRP. Diastolic blood pressure, however, significantly decreased by 3 mm Hg.[16]

Egert et al.[14•] studied the effects of increased intake of ALA, EPA, and DHA in a 6-week parallel trial in 74 German normolipidemic men and women aged 19 to 43 years. Participants were randomly assigned to trial margarines that provided additional ALA (3.4 g/d), EPA (2.2 g/d), or DHA (2.3 g/d). No placebo group was included. In the ALA group, the ALA content of LDL particles increased by 178% and the EPA content increased by 36%, whereas DHA remained unchanged. Serum total and LDL cholesterol were not affected by the different treatments. Fasting serum triglycerides significantly decreased with EPA (-0.14 mmol/L), DHA (-0.30 mmol/L), and ALA (-0.17 mmol/L). DHA intake significantly increased serum HDL cholesterol, whereas no changes were found with ALA or EPA intake.[14•]

Finally, Dodin et al.[18••] evaluated the effect of flaxseed on markers of cardiovascular disease risk in 169 healthy menopausal Canadian women who were randomly assigned to 40 g/d of flaxseed-based products or wheat-based products for 12 months. In the active-treatment group, ALA intake was increased by 8.8 g/d. Flaxseed products increased plasma ALA by 85% and plasma EPA by 51% after 12 months of intake, which differed significantly from wheat-based intervention (12% and 29%, respectively). Changes in plasma DHA were similar in both groups. The 12-month intervention with flaxseed had significant effects on body weight (-0.8 kg) and serum total cholesterol (-0.20 mmol/L) and a small adverse effect on HDL cholesterol (-0.08 mmol/L). LDL cholesterol was reduced by 0.13 mmol/L (P=0.09). Apolipoproteins A-I and B increased in both groups, but less during flaxseed-based than wheat-based intervention. Treatment had no effect on plasma lipoprotein(a), fibrinogen, CRP, insulin, glucose, or LDL peak particle size. The effect of ALA on plasma triglycerides was not reported.[18••]

Observational studies on ALA intake and cardiovascular risk

Zatónski et al.[2] examined trends in CHD mortality in 11 Eastern European countries and linked these figures to national data on vegetable oil consumption after 1990. They showed the strongest decline in CHD mortality in countries where rapeseed oil (9% ALA) rather than sunflower oil (0% ALA) was used. Although these data are suggestive for a beneficial effect of ALA, no definite conclusions can be drawn because the countries also differed in many other aspects that could impact cardiovascular health. Cross-country comparisons based on aggregate data cannot be adjusted for potential confounders, (eg, socioeconomic status, lifestyle and other dietary components) and should be considered for hypothesis generation only.

In Costa Rica, soybean oil has been substituted for palm oil since the 1980s, which has led to an increase in ALA intake. Campos et al.[19••] performed a case-control study in 3,638 Costa Rican individuals to examine the association between ALA and nonfatal MI (**Figure 3.1**). Participants were matched for age, sex, and area of residence. ALA intake was assessed by a 135-item food frequency questionnaire and ranged from 1.1 to 2.4 g/d (mean, 1.6 g/d) in this population. Dietary ALA was inversely associated with nonfatal MI, with odds ratios indicating a 39% reduced risk for approximately a 0.6-g/d difference in intake. The relationship between ALA and MI was nonlinear and mainly confined to the lowest levels of intake. Dietary ALA intake in this study correlated well with ALA in adipose tissue, plasma, and erythrocytes, but poorly with biomarkers of EPA and DHA, suggesting a direct cardioprotective effect of ALA rather than via conversion to long-chain n-3 fatty acids.[19••]

An anti-inflammatory action of ALA has been proposed as an explanation for the inverse association with cardiovascular diseases found in the case-control study in Costa Rica,[19••] although negative findings on ALA and inflammatory markers have been reported by others.[9] Dai et al.[20•] examined habitual ALA intake and plasma concentrations of inflammatory markers, including IL-6 and sIL-6R, in 353 middle-aged men in the United States who were recruited from a twin

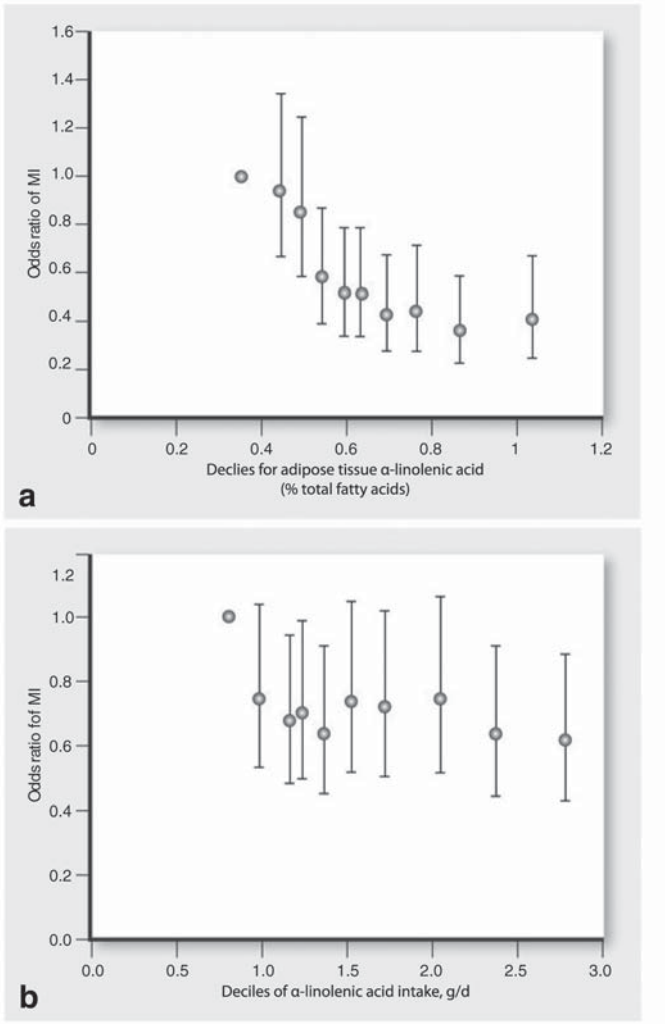

Figure 3.1 Odds ratios and 95% confidence intervals for nonfatal myocardial infarction (MI) by deciles of alpha-linolenic acid in adipose tissue **(a)** or intake **(b)** in a case-control study of 3,638 men and women in Costa Rica. Data were adjusted for smoking status, physical activity, household income, history of diabetes mellitus, history of hypertension, wait-to-hip ratio, saturated fat intake, and linoleic and *trans*-fatty acids in adipose tissue. (*From* Campos et al.,[19••] with permission.)

registry. ALA intake, assessed by the Willett questionnaire, ranged from 0.2 to 2 g/d, and was significantly inversely related to plasma sIL-6R independent of shared genetic factors and a wide range of potential confounders. A twin with a 1-g higher ALA intake (equivalent to one tablespoon of canola oil) had 11% lower sIL-6R concentrations than his twin with a low intake. Despite the robust association with sIL-6R, no significant associations were found with plasma IL-6, TNF-α, or high-sensitivity CRP.[20•] In a study of 511 Japanese employees of municipal offices,

ALA intake was assessed by a diet history questionnaire that had been validated against 16-day weighed food records (Pearson correlation for ALA, r=0.3).[21] ALA comprised 0.8% to 1.0% energy (>2 g/d) and was inversely related to serum CRP. Data were adjusted for age, body mass index (BMI), lifestyle factors, and physical activity, but not for dietary factors associated with ALA, and should therefore be interpreted with caution.

Smith et al.[22•] assessed dietary ALA intake by the Willett questionnaire in 260 post-MI patients and linked these data to 24-hour electrocardiogram recordings that were analyzed for ventricular premature beats. ALA intake (expressed per 1,000 kcal/d) was associated with a significant reduction in ventricular premature beats after adjustment for age, sex, cardiovascular medication, and co-morbidities. However, lifestyle (e.g., smoking) and dietary factors were not controlled for and the study had a cross-sectional design, which has inherent methodologic weaknesses.

Biomarker studies of ALA and cardiovascular risk

Previous analyses in the National Heart, Lung, and Blood Institute NHLBI Family Heart Study suggested that ALA may protect against atherosclerosis.[23,24] Recently, Sala-Vila et al.[25] investigated whether serum phosphatidylcholine content of ALA and other fatty acids was related to carotid atherosclerosis in a cross-sectional study of 451 asymptomatic Spanish individuals (mean age of 45 years) with primary dyslipidemia. Over half of the participants were treated with lipid-lowering drugs. The habitual Mediterranean diet in this population provided a mean ALA intake of 0.8 g/d, mainly from olive oil and walnuts, which correlated well with serum ALA (r=0.44). EPA and DHA intake from fish was 1.05 g/d on average. The association of serum fatty acids with intima-media thickness (IMT) of the carotid arteries was assessed, adjusting for age, sex, BMI, smoking, antihypertensive drugs, statins, and other serum fatty acids. Significant inverse associations were found between serum ALA and internal carotid artery IMT, and between serum DHA and common carotid artery IMT. Serum EPA was not associated with IMT.[25] In a small cross-sectional study of 50 Asian men and women (mean age of 58 years) who suffered a first nonfatal MI, the mean common carotid IMT was inversely associated with ALA content of erythrocytes (P=0.09) and ALA intake (P=0.02).[26] Data, however, were only adjusted for age, sex, and total energy (for dietary ALA). EPA and DHA were not consistently related to IMT. The average intake of ALA was 0.6 g/d, which was similar to that of EPA and DHA intake.

Ebbesson et al.[27] examined whether ALA content of erythrocytes was related to heart rate as a risk indicator for ventricular arrhythmias. They performed a cross-sectional study in 707 Alaskan Eskimos (mean age of 50 years) who had a habitual intake of marine n-3 fatty acids of 2.9 g/d. After adjustment for gender, height, BMI, blood pressure, smoking, and heart rate-lowering medications, no association was observed with ALA (P=0.98). EPA and DHA, on the other hand, were significantly inversely associated with heart rate.

The delta(6)-desaturase enzyme is the rate-limiting step in the conversion of ALA into EPA and DHA, and genetic variation in the delta(6)-desaturase gene (FADS2) may therefore affect the associations of ALA with cardiovascular health. Truong et al.[28], in a cross-sectional study, examined the association of ALA in adipose tissue with metabolic syndrome, and possible effect modification by FADS2. In a cohort of 1,815 men and women from Costa Rica, the prevalence of metabolic syndrome (656 cases) was 19% lower in the upper compared with the lower quintile of adipose tissue ALA. There was no association between ALA and metabolic syndrome among homozygous carriers of the FADS2 deletion allele, suggesting that conversion of ALA into EPA may play a role.[28] It should be noted, however, that the FADS2 polymorphism did not influence the inverse association of ALA with nonfatal MI in a previous case-control study from Costa Rica.[29]

Biomarker studies of ALA and cardiovascular endpoints

ALA status has been inversely associated with cardiovascular disease events, although data are less consistent than for EPA and DHA.[7-10] An overview of ALA biomarker studies is provided in **Table 3.2**. In the aforementioned case-control study by Campos et al.,[19••] ALA was measured in adipose tissue as a biomarker of intake, which correlated well with dietary ALA. ALA was strongly inversely associated with nonfatal MI, with a 57% reduced risk when comparing the 7th decile with the lowest decile. The relationship between ALA and MI was nonlinear and mainly confined to levels below the median (**Figure 3.1**). Mozaffarian et al.[30] suggested that ALA may particularly reduce CHD risk when intake of marine n-3 fatty acids is low. In the study by Campos et al.,[19••] however, concurrent fish intake (range, 3-32 g/d) or EPA and DHA intake (range, 130-520 mg/d) did not modify the associations of ALA with MI.

Yamagishi et al.[31] examined the prospective association of plasma fatty acids with risk of heart failure. The data used were from 3,575 white men and women in the United States (mean age of 54 years) who participated in the Atherosclerosis Risk in Communities (ARIC) study. During 14 years of follow-up, 195 cases of heart failure developed. Concentrations of n-3 fatty acids were assessed in plasma cholesteryl esters and in phospholipids. Plasma ALA was not associated with risk of heart failure (hazard ratios of 0.99 and 0.97 for upper versus lower quintiles of cholesteryl ester and phospholipid fractions, respectively; P=0.8). Plasma EPA also showed no association. Plasma DHA was inversely associated with incident heart failure, but only in women. Data were extensively adjusted for major confounders, including age, sex, BMI, lifestyle factors, and cardiovascular risk factors, but not for potential dietary confounders.[31]

Warensjö et al.[32] conducted a prospective cohort study of ALA in serum cholesteryl esters and cardiovascular mortality in more than 2,000 Swedish men (mean age of 50 years). The study had 30 years of follow-up and comprised over 60,000 person-years. A 10% higher risk of fatal cardiovascular events was found per standard deviation increase in serum ALA, which was borderline statistically significant. Data were adjusted for serum cholesterol, BMI, smoking, physical activity and presence of hypertension.

Table 3.2 Overview of biomarker studies of alpha-linolenic acid and cardiovascular events published between January 2008 and June 2010

Study	Year	Population	Design	Outcome
Campos et al.[19••]	2008	Costa Rica: 1,891 cases with first nonfatal MI and 1,891 population-based controls; matching for age, sex, and area of residence	Case-control study: association of ALA intake from FFQ and ALA in adipose tissue with risk of first nonfatal MI	OR (95% CI) for first nonfatal MI was 0.41 (0.25-0.67) for top vs lowest decile of ALA in adipose tissue, and 0.61 (0.42-0.88) for high vs low ALA intake; associations only present at lower ALA levels
Yamagishi et al.[31]	2008	USA, Minneapolis: 3,575 white men and women from ARIC study, ages 45-64 y	Prospective cohort study: association of plasma ALA with incident heart failure; 14.3 y of follow-up	195 participants (5.5%) developed heart failure; ALA status (top vs bottom quintile) was not associated with incident heart failure; age- and sex-adjusted HR was 0.99 (0.63-1.53) for cholesteryl ester fraction and 0.97 (0.61-1.54) for phospholipid fraction
Warensjö et al.[32]	2008	Sweden: 2,009 men from ULSAM study, aged 50 y	Prospective cohort study: association of ALA in serum cholesteryl esters with CVD mortality; 30.7 y of follow-up	Multivariable-adjusted HR was 1.10 (1.00-1.21) per 1-SD increase in serum ALA
Park et al.[33]	2009	South Korea: 40 cases of ischemic stroke, 40 cases of hemorrhagic stroke and 40 healthy controls; matching for age and sex	Case-control study: association of ALA in erythrocytes with risk of ischemic and hemorrhagic stroke	Erythrocyte ALA concentrations (area %) in hemorrhagic stroke patients (0.71±0.21) and ischemic stroke patients (0.24±0.03) were not significantly different from controls (0.44±0.05) after adjustment for family history of stroke; inverse association of ALA with ischemic stroke after adjustment for age and systolic blood pressure (P=0.045).
Virtanen et al.[34••]	2009	Finland: 2,174 men from Kuopio Ischemic Heart Disease Risk Factor Study, ages 42-60 y	Prospective cohort study: association of serum ALA with incident atrial fibrillation; 17.7 y of follow-up	240 men (11.0%) developed atrial fibrillation; multivariable-adjusted HR for serum ALA (compared to Q1) was Q2: 1.26 (95% CI: 0.84-1.89), Q3: 0.74 (0.46-1.20), and Q4: 1.14 (0.72-1.79; P-trend = 0.98).
Lemaitre et al.[35••]	2009	USA, Seattle: 265 out-of-hospital sudden cardiac arrest patients and 415 community members; matching for age, sex, and calendar year	Case-control study: association of ALA in erythrocytes with risk of sudden cardiac death; blood collection immediately after the event (patients) or during interview (control)	Multivariable-adjusted OR over quartiles of ALA in erythrocytes (compared to Q1): Q2 was 1.7 (1.0-3.0), Q3 was 1.9 (1.1-3.3), Q4 was 2.5 (95% CI, 1.3-4.8); association independent of erythrocyte levels of EPA and DHA, linoleic acid, and *trans*-fatty acids

Abbreviations: ALA, alpha-linolenic acid; ARIC, Atherosclerosis Risk in Communities; CVD, cardiovascular diseases; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFQ, food frequency questionnaire; HR, hazard ratio; MI, myocardial infarction; OR, odds ratio; Q, quartile; SD, standard deviation; ULSAM, Uppsala Longitudinal Study of Adult Men.

Erythrocyte concentrations of n-3 fatty acids were studied in relation to hemorrhagic and ischemic stroke in a case-control study of 120 Asian men and women (mean age of 57 years).[33] The average total n-3 fatty acid intake in this population was 1.1 g/d, about half of which was ALA. Erythrocyte ALA concentrations (area%) were 0.71±0.21 in hemorrhagic stroke patients and 0.24±0.03 in ischemic stroke patients, which did not differ significantly from controls (0.44±0.05) after adjustment for family history of stroke. In a logistic regression model, both ALA, EPA and DHA were significantly inversely related to ischemic stroke. EPA and DHA were also inversely related to hemorrhagic stroke, but this was not the case for ALA. Data, however, were only adjusted for age and systolic blood pressure, so residual confounding may have been present.

The risk of atrial fibrillation in relation to serum n-3 fatty acids was examined in a prospective cohort study of 2,174 Finnish men (mean age of 53 years).[34••] During 18 years of follow-up, 240 events of atrial fibrillation occurred. Serum ALA was not associated with incidence of atrial fibrillation. Hazard ratios in consecutive quartiles of ALA were 1.26 (95% CI: 0.84-1.89), 0.74 (95% CI: 0.46-1.20), and 1.14 (95% CI: 0.72-1.79) compared with the lowest quartile (*P*-trend = 0.98). Serum EPA did not show an association but DHA was inversely related to atrial fibrillation (hazard ratio of 0.62 for upper vs lower quartile; *P*-trend = 0.02).[34••]

Finally, Lemaitre et al.[35••] investigated in a case-control study whether ALA levels in erythrocytes were associated with risk of sudden cardiac death. Blood was collected from 265 out-of-hospital sudden cardiac arrest patients in Seattle, WA immediately after the event and from 415 randomly selected community members (mean age of 58 years). In contrast to what was expected, higher ALA levels were associated with a higher risk of sudden cardiac arrest. After adjustment for age, sex, smoking, diabetes, hypertension, education, physical activity, weight, height, and total fat intake, the risk increased over the quartiles with an odds ratio of 2.5 (95% CI: 1.3-4.8) for the highest compared with the lowest quartile. The association was independent of other fatty acids in erythrocyte membranes, including EPA and DHA.[35••]

Conclusions

The trials reviewed here consistently showed an increase in blood ALA levels after ALA supplementation, starting at low doses (<2 g/d). ALA supplementation also increased blood levels of EPA, but not of DHA, indicating conversion of ALA to EPA through elongation and desaturation. Short-term trials (6-12 weeks) in generally healthy individuals mostly showed no or inconsistent effects of ALA intake (1.2-3.6 g/d) on blood lipids, LDL oxidation, lipoprotein(a), and apolipoproteins A-I and B. Previous studies suggested an anti-inflammatory action of ALA,[5,7,8] but recent trials showed little effect of ALA on CRP or other inflammatory markers. There was, however, an interesting inverse association of ALA with sIL-6R in the twin study by Dai et al.,[20•] which warrants further investigation. The few studies that addressed ALA intake in relation to

glucose metabolism or blood pressure yielded inconsistent results.[16,17,18••] There is, however, some observational evidence that a high ALA status may be related to a lower risk of metabolic syndrome.[28] Long-term treatment with high ALA doses had a beneficial effect on body weight and blood LDL cholesterol,[18••] which warrants confirmation in future trials.

Previous evidence favored recommendations for modest dietary consumption of ALA (2-3 g/d) for the primary and secondary prevention of CHD.[7] The recently published case-control study by Campos et al.[19••] showing strong inverse associations of ALA status and intake with nonfatal MI is in line with this recommendation. Recent data provide some support that ALA could protect against atherosclerosis,[25,26] but it should be noted that this evidence comes from relatively small cross-sectional studies and that conclusions on causality cannot be drawn. Concerning the hypothesis of an anti-arrhythmic effect of n-3 fatty acids, there was some evidence for protection against ventricular premature beats,[22•] but no evidence for a relation with heart rate[27] or incidence of atrial fibrillation.[34••] Moreover, data from two recent well-conducted epidemiological studies suggested that high tissue ALA is related to an increased rather than decreased risk of fatal cardiovascular events[32] and sudden death.[35••] Harris et al. performed a meta-analysis of previous studies on tissue fatty acid composition and risk of CHD published until 2006. They showed that ALA in adipose tissue or phospholipids was inversely associated with CHD, although the association was not statistically significant for fatal events.[36] Of 16 studies reviewed, about half were supportive for a beneficial role of ALA, whereas other studies were negative or supportive for an adverse effect.[36]

In conclusion, there is a need for long-term trials investigating the effect of ALA supplementation on cardiovascular risk factors and clinical end points. Data are awaited from the Alpha Omega Trial, in which 4,837 post-MI patients (mean age of 69 years) were randomized to 2 g/d of ALA and/or 400 mg/d of EPA and DHA and followed for fatal and nonfatal cardiovascular disease events for 40 months.[37] The findings of this study may answer the question of whether a food-based dose of ALA affects cardiovascular health in high-risk individuals and, if so, whether this is comparable to the effect of marine n-3 fatty acids.

Disclosures

Dr. Geleijnse's institution received unrestricted grants from the Dutch Product Board for Margarine Fats and Oils and from the Alpro Foundation for an epidemiologic study of fatty acid intake and cardiovascular diseases in the Dutch population. It also received an unrestricted grant from Unilever to conduct the Alpha Omega Trial (http://www.alphaomegatrial.com). No other potential conflicts of interest relevant to this article were reported.

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- •• Of major importance

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This is a well-controlled double-blind dietary intervention study in which 79 healthy adults consumed ALA (3.4 g/d), EPA (2.2 g/d), and DHA (2.4 g/d) via enriched margarines for 6 weeks. Serum total and LDL cholesterol were not affected by treatment. Fasting serum triglycerides significantly decreased with EPA (-0.14 mmol/L), DHA (-0.30 mmol/L), and ALA (-0.17 mmol/L).

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    This is a large, long-term food-based trial in 169 women who were randomly assigned to receive 40 g/d of flaxseed-based products or wheat-based products for 12 months. In the active-treatment group, ALA intake was increased by 8.8 g/d. Flaxseed had significant effects on body weight (-0.8 kg) and serum total and LDL cholesterol (-0.20 and -0.13 mmol/L, respectively) and a small adverse effect on HDL cholesterol (-0.08 mmol/L). The high ALA dose had no effect on inflammatory markers.

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    In this study, 1,819 patients who survived an MI and 1,817 matching controls provided adipose tissue for analysis of ALA and completed a validated food questionnaire. ALA in adipose tissue ranged from 0.36% in the lowest decile to 1.04% in the highest decile. The corresponding median ALA intakes were 1.1 to 2.4 g/d. The risk of nonfatal MI was strongly reduced to adipose tissue ALA levels of about 0.7%, which corresponds to an intake of about 1.8 g/d. Further increases in intake were not associated with increased protection.

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    In this cross-sectional study, habitual ALA intake was examined in relation to plasma inflammatory markers, including IL-6 and sIL-6R, in 353 middle-aged male twins. ALA intake ranged from 0.2 to 2 g/d and was significantly inversely associated with plasma sIL-6R, but not with plasma IL-6 or other inflammatory markers. A major strength of this study is the use of twins who share genetic factors and who generally have similar lifestyles and diets.

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Data from clinical trials raised the hypothesis of a protective effect of marine n-3 fatty acids against ventricular arrhythmias in CHD patients. Whether ALA could also prevent arrhythmia, however, is not known. In this cross-sectional study, ALA intake was assessed in 260 post-MI patients and linked to electrocardiogram recordings. ALA was inversely associated with ventricular premature beats, after adjustment for age, sex, cardiovascular medication, and co-morbidities.

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This study assessed the relationship between serum concentrations of n-3 fatty acids and risk of atrial fibrillation in 2,174 Finnish men who were followed for almost 18 years. Only serum DHA was associated with the risk, with a 38% lower risk in the highest quartile. Serum ALA was not associated with incident atrial fibrillation, not even when serum EPA and DHA were low.

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Previous population-based case-control studies of this research group showed strong inverse associations of dietary intake and erythrocyte membrane levels of marine n-3 fatty acids with the risk of sudden cardiac death. In this article, data from a similar case-control study of ALA in erythrocyte membranes and sudden cardiac arrest are reported. Blood was obtained at the time of cardiac arrest (cases) or at the time of an interview (controls). Against expectations, higher membrane ALA was associated with a increased risk of sudden cardiac arrest, with an odds ratio of 2.5 (95% CI: 1.3-4.8) for the upper versus lower quartile.

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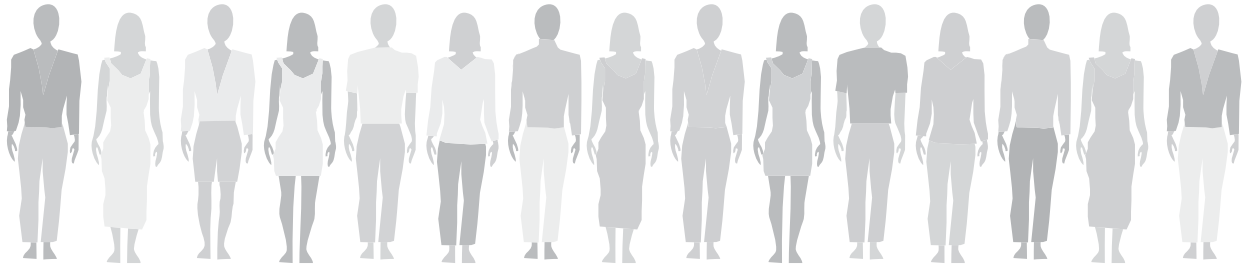

4

Alpha-linolenic acid intake and 10-year incidence of coronary heart disease and stroke in 20,000 middle-aged men and women in the Netherlands

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Abstract

Background: Whether intake of alpha-linolenic acid (ALA), the plant-derived n-3 polyunsaturated fatty acid (PUFA), could prevent cardiovascular diseases is not yet clear. We examined the associations of ALA intake with 10-year incidence of coronary heart disease (CHD) and stroke in the Netherlands.

Methods: Data were collected from a general population of 20,069 generally healthy men and women, aged 20 to 65 years. Habitual diet was assessed at baseline (1993-1997) with a validated 178-item food frequency questionnaire. Incidences of CHD and stroke were assessed through linkage with mortality and morbidity registers. Hazard ratios (HR) were calculated with multivariable Cox proportional hazards models, adjusted for age, gender, lifestyle, and dietary factors.

Results: During 8–13 years of follow-up, we observed 280 incident CHD events (19% fatal) and 221 strokes (4% fatal). Intakes of energy-adjusted ALA in quintiles ranged from less than 1.0 g/d in the bottom quintile (Q1) to more than 1.9 g/d in the top quintile (Q5). ALA intake was not associated with incident CHD, with HRs varying between 0.89 and 1.01 (all $P>0.05$) in Q2-Q5 compared with the bottom quintile of ALA intake. For incident stroke, however, participants in Q2-Q5 had a 35-50% lower risk compared with the reference group. HRs (95% CI) were 0.65 (0.43-0.97), 0.49 (0.31-0.76), 0.53 (0.34-0.83), and 0.65 (0.41-1.04) for Q2-Q5 respectively.

Conclusion: In this general Dutch population, ALA intake was not associated with incident CHD. The data suggested that a low intake of ALA may be a risk factor for incident stroke. These results warrant confirmation in other population-based studies and in trials.

Introduction

Numerous studies suggest that marine n-3 polyunsaturated fatty acids (PUFA), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), protect against cardiovascular diseases (CVD).[1-3] However, the role of the plant-derived n-3 PUFA alpha-linolenic acid (ALA) in CVD prevention is less clear.[4-6] ALA is mainly found in vegetable oils such as soybean, canola, and flaxseed oil, and walnuts.[7]

In Western countries such as the Netherlands, the intake of ALA is 5-10 times higher than n-3 PUFA from fish.[8] ALA is an essential fatty acid, which means that humans have to obtain it through their diet. Humans can convert ALA into the very-long-chain fatty acids EPA and DHA, although conversion only occurs to a limited extent.[9,10] Apart from potential indirect effects of ALA on CVD via conversion into EPA and DHA, it is suggested that ALA could have direct anti-inflammatory,[6,11] anti-arrhythmic,[12] anti-thrombotic,[12,13] or neuroprotective effects.[14] However, others concluded that there was insufficient evidence that ALA influences risk factors for CVD.[15,16]

Several prospective cohort studies showed inverse associations of ALA intake with fatal CVD,[17] fatal coronary heart disease (CHD),[17-19] sudden cardiac death,[12] incident CHD,[20] incident myocardial infarction (MI),[21] or nonfatal MI.[20] Other cohort studies suggested no protection of ALA intake against fatal CVD,[22] fatal CHD,[12,21,23] sudden death,[20] incident CHD,[23] or nonfatal MI.[12,18] The relation of ALA intake with fatal CHD has been summarized in a meta-analysis of 5 prospective cohort studies showing that ALA intakes of around 2 g/d were associated with a 21% lower risk of fatal CHD (relative risk: 0.79; 95% CI: 0.60-1.04), compared with intakes of 0.8 g/d.[24]

Little is known about the association of ALA intake with stroke. In a nested case-control study in 192 American middle-aged men, serum ALA was inversely associated with stroke,[25] although this was not confirmed in a Japanese nested case-control study of with 197 cases of hemorrhagic and ischemic stroke.[26] However, Japanese have higher mortality from stroke and have higher serum levels of n-3 PUFA compared with white Americans and Europeans, which makes it difficult to compare the results.[26]

We examined the 10-year incidence of CHD and stroke in relation to ALA intake in a population-based cohort of over 20,000 adults in the Netherlands.

Methods

Ethical statement

This research was performed in accordance with the ethical principles for medical research involving human subjects outlined in the Declaration of Helsinki. This research was approved by the Medical Ethics Committee of TNO Prevention and Health (Leiden, The Netherlands). All participants gave written informed consent.

Design and study population

The "Monitoring Project on Risk Factors for Chronic Diseases" (MORGEN) study is a Dutch population-based cohort of 22,654 men and women, aged 20 to 65 years. MORGEN is part of the European Prospective Investigation into Cancer and Nutrition (EPIC) study.[27] For the current study we excluded participants who did not provide informed consent for vital status follow-up (n=701). We also excluded 72 participants without dietary information and 97 participants with extreme energy intakes (<500 or >4,500 kcal for women and <800 or >5,000 kcal for men). Furthermore, participants with a history of MI or stroke at baseline were excluded (n=442). We also excluded participants who reported use of lipid or blood pressure lowering medication (n=1,093) and 180 participants with diabetes resulting in 20,069 participants (8,988 men and 11,081 women).

Dietary assessment

The habitual diet was assessed at baseline with the Dutch EPIC Food Frequency Questionnaire (FFQ) a self-administered 178-item FFQ covering the previous year.[28,29] The FFQ included foods that covered the intake of foods and nutrients relevant to chronic disease etiology for at least 90% of the national mean intake. Participants indicated consumption of main food groups in times per day, per week, per month, per year, or as never, combined with questions on the relative intakes of foods within food groups (seldom/never, sometimes, often, mostly/always). In addition, we calculated raw vegetable consumption as the sum of lettuce, cucumber, tomato, carrots, cabbage, sweet pepper, and chicory consumption in grams per day, because these raw vegetables are consumed together with salad dressings, which is a main source of ALA.

Nutrient intakes were calculated with the "Dutch food composition table" of 1998. For individual fatty acids, we used the table of 2001. All nutrients were adjusted for total energy intake with the residuals method.[30] The Dutch EPIC questionnaire has been validated for several food groups and nutrients. The reproducibility (estimated by 2 repeated measurements) and the relative validity (intake assessed by the FFQ compared to intakes assessed by 12 monthly 24-h recalls) of the FFQ for various food groups and nutrients were assessed among 121 Dutch men and women.[28,29] The Spearman rank correlations for the reproducibility of the FFQ after 6 months were 0.90 and 0.80 for total energy and 0.83 and 0.77 for total fat in men and women respectively. The relative validity of the FFQ was 0.77 and 0.62 for total energy and 0.74 and 0.63 for total fat in men and women respectively.

Mortality and morbidity

Vital status was checked through linkage with the national population register. Participants were followed for the occurrence of CVD by linkage with Statistics Netherlands for cause-specific

mortality and to the national hospital discharge register for nonfatal events by a validated probabilistic linkage method described in detail elsewhere.[31] Incident CHD included fatal CHD (I20-I25), fatal and nonfatal cardiac arrest (I46), and nonfatal MI (I21-I22) according to the International Classification of Diseases (ICD-10, WHO). Incident stroke included fatal and nonfatal cerebrovascular accidents and transient ischemic attacks (I60-I66, G45). For hospital admissions, corresponding ICD9 codes were used. Both primary and secondary causes of death were used for the classification of fatal events. For nonfatal events we used the primary indication for hospital admission. Participants were followed until death, incident CHD or stroke (first events only), date of loss-to-follow-up (n=693) or 1 January 2006, whichever came first.

Other baseline characteristics

Body weight, height, and blood pressure were measured by trained research nurses. Levels of total cholesterol and high-density lipoprotein cholesterol were assessed in plasma (non-fasting).[32] Questionnaires were used to assess presence of diabetes, history of MI, history of stroke, medication use, parental history of MI (MI of father before the age of 55 year or MI of mother before the age of 65 years), educational level, and cigarette smoking. Alcohol intake (assessed by FFQ) was categorized as no intake, low to moderate intake (men ≤2 and women ≤1 glasses/d), or high intake (men >2 and women >1 glasses/d). Physical activity was assessed with a validated questionnaire in 76% of our cohort (from 1994 onwards).[33] For this subset, we calculated whether participants were engaged in activities with a metabolic equivalent score ≥4 (yes/no). Cycling (yes/no) and sports (yes/no) were previously shown to be inversely related to CVD incidence in this study population.[34]

Statistical analysis

Participants' characteristics by quintiles of energy-adjusted ALA intake are presented as means with SD, medians with interquartile ranges, or percentages. Correlations between the energy-adjusted intakes of different types of fatty acids were assessed with the Spearman rank correlation test.

We used Cox proportional hazards models to estimate relative risks for the incidence of CHD, total stroke, and ischemic stroke across quintiles of energy-adjusted ALA intake at baseline. For hemorrhagic stroke we had insufficient cases. Hazard ratios (HR) with 95% confidence intervals (CI) were obtained with the bottom quintile of ALA intake as the reference category. The proportional hazards assumption was tested and not rejected based on Schoenfeld residuals and visual inspection.

In model 1, we adjusted for age and gender. In model 2, we additionally adjusted for total energy intake (kJ/d), body mass index (kg/m^2), alcohol intake (no, low to moderate, or high),

current cigarette smoking, high educational level (completed higher vocational training or university), parental history of MI. In model 3, we added energy-adjusted intakes of vitamin C (mg/d), beta-carotene (µg/d), fiber (g/d), saturated fatty acids (g/d), *trans*-fatty acids (g/d), and polyunsaturated fatty acids other than ALA (g/d).

Possible confounding by physical activity was checked in the subgroup of participants with information on physical activity. We examined whether further adjustment for systolic blood pressure and total cholesterol changed the association of ALA with CHD and stroke to assess whether these factors could be intermediates. Effect modification was evaluated for age and gender. In various foods ALA is highly correlated with saturated fatty acids and *trans*-fatty acids. We therefore separately analyzed ALA intake from salad dressings (mayonnaise + soy bean oil), with a low content of saturated fatty acids and *trans*-fatty acids vs. ALA intake from other sources, mutually adjusted. These analyses were additionally adjusted for the intake of raw vegetables. All statistical analyses were performed with SAS (version 9.1; SAS Institute). Two-sided *P*-values <0.05 were considered statistically significant.

Results

Population characteristics

Participants were on average ± SD 41.5 ± 11.1 years at baseline, and 45% were male. Men had higher ALA intakes than women (1.6 ± 0.6 vs. 1.2 ± 0.5 g), but values were similar after energy adjustment (1.4 ± 0.4 g). During 8-13 years of follow-up (median 10.5 y), 280 CHD events (19% fatal) and 221 strokes (4% fatal) occurred. Total stroke comprised 80 cases of ischemic cerebrovascular accident (36%), 59 transient ischemic attacks (27%), 47 cases of hemorrhagic stroke (21%), and 35 cases of unspecified stroke (16%).

The main sources of ALA intake were mayonnaise (15%), margarine (14%), soy bean oil (8%), and bread (8%). Median energy-adjusted ALA intakes in quintiles ranged from less than 1.0 g/d to more than 1.9 g/d. ALA intake was positively associated with intakes of total PUFA (mainly linoleic acid), *cis*-monounsaturated fat, *trans*-fatty acids, and saturated fatty acids, but not with EPA-DHA (**Table 4.1**). The Spearman rank correlations with ALA were 0.54 for linoleic acid, 0.41 for *cis*-monounsaturated fatty acids, 0.22 for *trans*-fatty acids, and 0.18 for saturated fatty acids.

ALA intake and incident CHD and stroke

After adjustment for potential confounders, ALA intake was not associated with incident CHD. HRs varied between 0.89 and 1.01 (all $P > 0.05$) compared with the bottom quintile of ALA intake (**Table 4.2**). ALA intake was inversely associated with total stroke (**Figure 4.1**) and ischemic stroke

Table 4.1 Baseline characteristics of 20,069 Dutch men and women, aged 20-65 years, by quintiles of energy-adjusted ALA intake[a]

	Quintiles of ALA intake				
	Q1	Q2	Q3	Q4	Q5
n	4,013	4,014	4,014	4,014	4,014
ALA					
g/d	0.9 ± 0.2	1.2 ± 0.1	1.3 ± 0.05	1.5 ± 0.1	2.0 ± 0.4
en%	0.4 ± 0.05	0.4 ± 0.03	0.5 ± 0.02	0.6 ± 0.04	0.8 ± 0.1
from dressings, g/d	0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.4 ± 0.2	0.7 ± 0.5
from other sources, g/d	0.8 ± 0.2	0.9 ± 0.1	1.0 ± 0.2	1.1 ± 0.2	1.3 ± 0.4
Linoleic acid					
g/d	11.1 ± 4.3	12.5 ± 3.2	13.4 ± 3.0	14.6 ± 3.1	17.2 ± 4.2
en%	4.4 ± 1.4	4.8 ± 1.3	5.2 ± 1.2	5.8 ± 1.2	6.7 ± 1.5
EPA-DHA,[b] mg/d	114 (61-194)	110 (60-192)	111 (58-189)	112 (61-194)	117 (65-198)
Male gender, %	59	41	38	40	46
Age, y	41.8 ± 11.7	42.0 ± 11.2	41.8 ± 11.0	41.3 ± 10.9	40.6 ± 10.6
Body mass index, kg/m^2	24.9 ± 3.7	24.9 ± 3.8	24.8 ± 3.8	24.8 ± 3.8	24.9 ± 4.1
PUFA					
g/d	13.9 ± 4.40	15.9 ± 3.2	17.0 ± 3.0	18.4 ± 3.1	21.6 ± 4.3
en%	5.6 ± 1.5	6.1 ± 1.3	6.6 ± 1.2	7.3 ± 1.2	8.4 ± 1.5
Cis-MUFA					
g/d	27.5 ± 5.9	30.0 ± 4.7	31.1 ± 4.6	32.0 ± 4.7	34.0 ± 5.8
en%	10.9 ± 2.0	11.5 ± 1.9	12.0 ± 1.9	12.5 ± 1.9	13.3 ± 2.0
TFA					
g/d	3.4 ± 1.5	3.7 ± 1.2	3.8 ± 1.1	3.9 ± 1.2	4.1 ± 1.5
en%	1.4 ± 0.5	1.4 ± 0.5	1.5 ± 0.5	1.5 ± 0.5	1.6 ± 0.5
SFA					
g/d	35.1 ± 7.8	36.7 ± 6.1	37.3 ± 5.8	37.7 ± 5.8	38.3 ± 6.4
en%	13.8 ± 2.7	14.2 ± 2.5	14.4 ± 2.4	14.6 ± 2.4	14.9 ± 2.4
Carbohydrate, % of energy	45.4 ± 6.3	44.5 ± 5.8	43.5 ± 5.4	42.7 ± 5.2	41.3 ± 5.1
Protein, en%	15.0 ± 2.4	15.5 ± 2.3	15.3 ± 2.2	15.2 ± 2.1	14.4 ± 2.0
Vitamin C,[b] mg/d	103 (77-138)	101 (77-132)	99 (77-129)	98 (75-127)	93 (70-124)
Beta carotene,[b] mg/d	1.4 (1.1-1.7)	1.4 (1.1-1.8)	1.4 (1.2-1.8)	1.5 (1.2-1.9)	1.5 (1.2-2.0)
Fiber, g/d	24.3 ± 6.0	24.7 ± 5.1	24.7 ± 4.8	24.8 ± 4.9	24.5 ± 5.4
Energy intake, MJ/d	10.6 ± 2.9	9.0 ± 2.5	8.8 ± 2.5	9.1 ± 2.6	10.1 ± 2.9
Current smoking, %	34	33	35	38	44
Alcohol consumption, %					
No	12	13	12	12	13
Low to moderate	50	58	58	61	58
High	38	29	30	27	29
Highly educated,[c] %	24	25	27	25	24
Dutch ethnicity, %	97	97	97	96	95
Physically active,[d] %					
Engaged in cycling	59	61	60	59	57
Engaged in sports	39	40	38	35	34
Parental history of MI, %	8	9	9	10	9
Plasma total cholesterol,[e] mmol/l	5.3 ± 1.1	5.3 ± 1.0	5.3 ± 1.1	5.3 ± 1.1	5.2 ± 1.0
Plasma HDL-cholesterol,[e] mmol/l	1.3 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.3 ± 0.4
Systolic blood pressure, mm Hg	122.0 ± 15.7	120.1 ± 15.9	119.5 ± 15.5	119.0 ± 15.1	119.0 ± 15.3
Diastolic blood pressure, mm Hg	77.0 ± 10.4	76.2 ± 10.4	76.0 ± 10.5	75.7 ± 10.2	75.6 ± 10.3

Abbreviations: ALA, alpha-linolenic acid; en%, percent of energy; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Q1-Q5, quintiles; SFA, saturated fatty acids; MI, myocardial infarction; TFA, *trans*-fatty acids.

[a] Values are means ± SD, unless indicated otherwise.

[b] Median with interquartile range.

[c] University or higher vocational training.

[d] Available for participants enrolled between 1994 and 1997 (n=15,423).

[e] Nonfasting.

Table 4.2 Associations of incident coronary heart disease and stroke by quintiles of energy-adjusted ALA intake in 20,069 Dutch men and women[a]

	Quintiles of ALA intake, g/d				
	Q1	Q2	Q3	Q4	Q5
n	4,013	4,014	4,014	4,014	4,014
Median ALA, g/d	1.0	1.2	1.3	1.5	1.9
Coronary heart disease					
No. events	68	51	47	53	61
Model 1[b]	1.0 (ref)	0.90 (0.63-1.30)	0.87 (0.60-1.27)	1.01 (0.70-1.44)	1.16 (0.82-1.64)
Model 2[c]	1.0 (ref)	0.89 (0.61-1.29)	0.89 (0.61-1.30)	0.97 (0.67-1.40)	1.03 (0.72-1.46)
Model 3[d]	1.0 (ref)	0.89 (0.61-1.30)	0.90 (0.61-1.33)	0.97 (0.66-1.44)	1.01 (0.66-1.54)
Total stroke					
No. events	64	43	34	35	45
Model 1[b]	1.0 (ref)	0.71 (0.48-1.04)	0.57 (0.38-0.87)	0.62 (0.41-0.93)	0.83 (0.57-1.22)
Model 2[c]	1.0 (ref)	0.68 (0.46-1.01)	0.53 (0.34-0.81)	0.59 (0.39-0.90)	0.78 (0.53-1.15)
Model 3[d]	1.0 (ref)	0.65 (0.43-0.97)	0.49 (0.31-0.76)	0.53 (0.34-0.83)	0.65 (0.41-1.04)
Ischemic stroke					
No. events	45	27	21	23	28
Model 1[b]	1.0 (ref)	0.65 (0.40-1.05)	0.52 (0.31-0.87)	0.59 (0.36-0.98)	0.74 (0.46-1.20)
Model 2[c]	1.0 (ref)	0.63 (0.39-1.02)	0.45 (0.26-0.77)	0.55 (0.33-0.92)	0.70 (0.43-1.12)
Model 3[d]	1.0 (ref)	0.63 (0.38-1.04)	0.45 (0.26-0.79)	0.56 (0.32-0.97)	0.70 (0.39-1.26)

Abbreviations: ALA, alpha-linolenic acid; Q1-Q5, quintiles.

[a] Values are hazard ratios (95% CI), with the first quintile as the reference category.

[b] Model 1: adjusted for age and gender (n=20,069).

[c] Model 2: model 1 with additional adjustments for body mass index, total energy intake, cigarette smoking, educational level, parental history of myocardial infarction, alcohol intake (n=19,896).

[d] Model 3: model 2 with additional adjustments for intake of vitamin C, beta-carotene, fiber, saturated fatty acids, *trans*-fatty acids, polyunsaturated fatty acids other than ALA (n=19,896).

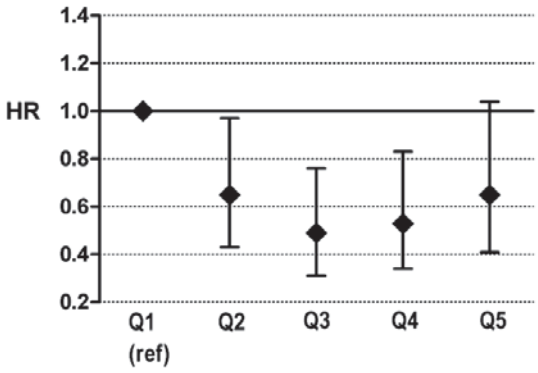

Figure 4.1 The association of incident total stroke by quintiles of energy-adjusted ALA intake.[a,b]

[a] Hazard ratios (95% CI) with the first quintile as the reference category, adjusted for age, gender, body mass index total energy intake, alcohol intake, cigarette smoking, education level, parental history of myocardial infarction, intake of vitamin C, beta-carotene, fiber, saturated fatty acids, *trans*-fatty acids, polyunsaturated fatty acids other than ALA.

[b] ALA, alpha-linolenic acid; HR, hazard ratio; Q1-Q5, quintiles.

incidence. Compared with the lowest quintile of ALA intake (<1.1 g/d), participants in the other quintiles had a 35-50% lower risk of incident total stroke and ischemic stroke. The lowest risks were observed in quintiles 3 and 4.

Median energy-adjusted intakes of ALA from salad dressings increased from 0.1 g/d to 0.7 g/d across quintiles. ALA from other sources (mainly margarines) increased from 0.7 to 1.4 g/d. Incident CHD was not associated with ALA from salad dressings (**Table 4.3**) or with ALA from other sources (**Table 4.4**). The inverse association of ALA intake with total and ischemic stroke was stronger for ALA from salad dressings compared with total ALA, while ALA from other sources was not associated with stroke. Compared with the bottom quintile of ALA intake,

Table 4.3 Associations of incident coronary heart disease and stroke by quintiles of energy-adjusted ALA intake from salad dressings in 20,069 Dutch men and women[a]

	Quintiles of ALA intake				
	Q1	Q2	Q3	Q4	Q5
n	4,013	4,014	4,014	4,014	4,014
Median ALA in salad dressings,[b] g/d	0.1	0.2	0.3	0.5	0.7
Median ALA in other sources, g/d	1.0	1.0	1.0	1.0	0.9
Coronary heart disease					
No. events	78	56	55	42	49
Model 1[c]	1.0 (ref)	0.93 (0.66-1.32)	1.07 (0.75-1.51)	0.90 (0.62-1.32)	1.20 (0.83-1.73)
Model 2[d]	1.0 (ref)	0.93 (0.66-1.32)	1.02 (0.71-1.45)	0.83 (0.56-1.23)	1.06 (0.73-1.54)
Model 3[e]	1.0 (ref)	0.95 (0.67-1.34)	1.04 (0.72-1.49)	0.86 (0.58-1.29)	1.14 (0.76-1.70)
Model 4[f]	1.0 (ref)	0.94 (0.66-1.34)	1.03 (0.71-1.49)	0.85 (0.56-1.30)	1.12 (0.72-1.75)
Total stroke					
No. events	78	60	28	26	29
Model 1[c]	1.0 (ref)	0.85 (0.60-1.19)	0.45 (0.29-0.69)	0.44 (0.28-0.70)	0.55 (0.36-0.86)
Model 2[d]	1.0 (ref)	0.83 (0.59-1.18)	0.44 (0.28-0.68)	0.41 (0.26-0.66)	0.52 (0.33-0.81)
Model 3[e]	1.0 (ref)	0.82 (0.57-1.16)	0.42 (0.27-0.66)	0.39 (0.24-0.62)	0.46 (0.28-0.74)
Model 4[f]	1.0 (ref)	0.85 (0.59-1.20)	0.45 (0.29-0.72)	0.44 (0.27-0.72)	0.57 (0.34-0.96)
Ischemic stroke					
No. events	54	37	20	17	16
Model 1[c]	1.0 (ref)	0.77 (0.50-1.17)	0.47 (0.28-0.79)	0.43 (0.24-0.74)	0.44 (0.25-0.78)
Model 2[d]	1.0 (ref)	0.74 (0.48-1.15)	0.45 (0.27-0.77)	0.40 (0.23-0.71)	0.41 (0.23-0.73)
Model 3[e]	1.0 (ref)	0.75 (0.48-1.16)	0.46 (0.27-0.79)	0.42 (0.23-0.74)	0.42 (0.23-0.79)
Model 4[f]	1.0 (ref)	0.78 (0.50-1.21)	0.50 (0.29-0.86)	0.47 (0.26-0.86)	0.51 (0.26-1.02)

Abbreviations: ALA, alpha-linolenic acid; Q1-Q5, quintiles.

[a] Values are hazard ratios (95% CI), with the first quintile as the reference category.

[b] Analyses on ALA in salad dressings are adjusted for ALA in other sources in all models.

[c] Model 1: adjusted for age and gender (n=20,069).

[d] Model 2: model 1 with additional adjustments for body mass index, total energy intake, cigarette smoking, educational level, parental history of myocardial infarction, alcohol intake (n=19,896).

[e] Model 3: model 2 with additional adjustments for intake of vitamin C, beta-carotene, fiber, saturated fatty acids, *trans*-fatty acids, polyunsaturated fatty acids other than ALA (n=19,896).

[f] Model 4: model 3 with additional adjustment for raw vegetables (n=19,896).

Table 4.4 Associations of incident CHD and stroke by quintiles of energy-adjusted ALA intake from other sources than salad dressings in 20,069 Dutch men and women[a]

	Quintiles of ALA intake				
	Q1	Q2	Q3	Q4	Q5
n	4,013	4,014	4,014	4,014	4,014
Median ALA in other sources,[b] g/d	0.7	0.9	1.0	1.1	1.4
Median ALA in salad dressings,[b] g/d	0.3	0.3	0.3	0.3	0.3
Coronary heart disease					
No. events	66	42	46	54	72
Model 1[c]	1.0 (ref)	0.72 (0.49-1.06)	0.81 (0.55-1.19)	0.87 (0.61-1.25)	0.96 (0.68-1.34)
Model 2[d]	1.0 (ref)	0.73 (0.49-1.10)	0.81 (0.54-1.22)	0.88 (0.61-1.29)	0.91 (0.64-1.29)
Model 3[e]	1.0 (ref)	0.73 (0.48-1.10)	0.80 (0.53-1.22)	0.85 (0.57-1.28)	0.85 (0.56-1.27)
Model 4[f]	1.0 (ref)	0.73 (0.48-1.10)	0.80 (0.53-1.22)	0.85 (0.57-1.28)	0.84 (0.56-1.27)
Total stroke					
No. events	41	38	38	45	59
Model 1[c]	1.0 (ref)	0.87 (0.56-1.36)	0.85 (0.54-1.33)	0.96 (0.62-1.47)	1.12 (0.75-1.67)
Model 2[d]	1.0 (ref)	0.91 (0.58-1.45)	0.88 (0.55-1.40)	0.97 (0.62-1.52)	1.10 (0.72-1.66)
Model 3[e]	1.0 (ref)	0.88 (0.55-1.41)	0.83 (0.51-1.35)	0.92 (0.57-1.48)	0.96 (0.59-1.56)
Model 4[f]	1.0 (ref)	0.88 (0.55-1.41)	0.82 (0.51-1.34)	0.89 (0.56-1.44)	0.93 (0.57-1.51)
Ischemic stroke					
No. events	29	26	22	26	41
Model 1[c]	1.0 (ref)	0.86 (0.51-1.47)	0.72 (0.41-1.26)	0.80 (0.47-1.36)	1.09 (0.68-1.77)
Model 2[d]	1.0 (ref)	0.85 (0.49-1.47)	0.69 (0.38-1.23)	0.77 (0.44-1.33)	1.02 (0.62-1.68)
Model 3[e]	1.0 (ref)	0.85 (0.49-1.50)	0.69 (0.38-1.27)	0.77 (0.42-1.39)	1.01 (0.56-1.83)
Model 4[f]	1.0 (ref)	0.85 (0.48-1.49)	0.68 (0.37-1.25)	0.75 (0.41-1.36)	0.98 (0.54-1.78)

Abbreviations: ALA, alpha-linolenic acid; Q1-Q5, quintiles.

[a] Values are hazard ratios (95% CI), with the first quintile as the reference category.

[b] Analyses on ALA from other sources than salad dressings are adjusted for ALA in salad dressings in all models.

[c] Model 1: adjusted for age and gender (n=20,069).

[d] Model 2: model 1 with additional adjustments for body mass index, total energy intake, cigarette smoking, educational level, parental history of myocardial infarction, alcohol intake (n=19,896).

[e] Model 3: model 2 with additional adjustments for intake of vitamin C, beta-carotene, fiber, saturated fatty acids, *trans*-fatty acids, polyunsaturated fatty acids other than ALA (n=19,896).

[f] Model 4: model 3 with additional adjustment for raw vegetables (n=19,896).

participants in the higher quintiles had an 18-61% lower risk of total stroke and a 25-58% lower risk of ischemic stroke. The inverse associations were most pronounced in quintiles 3 and 4. After additional adjustment for raw vegetable consumption, the associations were somewhat weaker, but remained statistically significant, except for the top quintile of ALA.

The associations of ALA intake with incident CHD and stroke did not differ in subgroups of age and gender. For the subgroup with information on physical activity (n=15,423), the full model with and without physical activity yielded similar results. Adjustment for plasma cholesterol or systolic blood pressure did not change the results. HRs (95% CI) for total stroke after additional inclusion of systolic blood pressure were 0.66 (0.44-0.98), 0.50 (0.32-0.78), 0.54 (0.34-0.84) and 0.67 (0.42-1.06) for Q2-Q5 compared with Q1, respectively.

Discussion

In this large prospective cohort study in the Netherlands, we found no association between ALA intake and incident CHD. However, ALA intakes >1.1 g/d were associated with a 35-50% lower risk of incident stroke, mainly ischemic stroke, compared with ALA intakes <1.1 g/d.

This study has several strengths, including almost complete mortality follow up and detailed information on potential confounders. Nonfatal cardiovascular events were assessed through the national hospital discharge register. In part of the subjects included in our analysis, hospital discharge diagnoses for MI were validated by comparison with the clinical registry of the Cardiology Department of the Maastricht University Hospital, showing a relatively high sensitivity (84%) and positive predictive value (97%) for MI.[35] We assume that misclassification of stroke was limited, because brain imaging is used to identify stroke and its subtypes in 98% of the patients admitted to Dutch hospitals.[36]

However, there were also limitations. First, misclassification of participants for ALA intake may have occurred. However, because we excluded participants with a history of MI or stroke, and participants who used cholesterol lowering or blood pressure lowering medication, we expect misclassification at baseline to be random rather than dependent on disease outcome. Second, hospital discharge diagnoses were assessed through probabilistic linkage with the national hospital discharge register. If we have missed events by this procedure, then this is unlikely to be related to ALA intake and may have caused bias towards the null.

In our study, ALA intakes in the range of 1.0-1.9 g/d were not associated with incident CHD. This is in line with a cohort study in elderly Dutch men (Zutphen Study), which did not show a benefit of ALA intake on incident CHD, for similar levels of intake.[23] In the Nurses' Health Study, with a difference of 0.7 g/d between the top and bottom quintile of ALA intake, ALA intake was inversely associated with fatal CHD, but not with nonfatal MI.[12,18] Our results on CHD differ from those of the Health Professionals Follow-up Study, in which an increase of one energy percent of linolenic acid (mainly ALA) intake was associated with a 60% lower risk of incident MI in men.[21] These results were not adjusted for other PUFA, saturated fatty acids, or *trans*-fatty acids, and the contrast between the top and bottom quintile of ALA intake was only 0.7 g/d (~0.3 energy percent). A later study of this cohort with additional adjustment for other fatty acids suggested a 16% lower risk for total CHD (borderline significant) corresponding with an increase of ALA of 1g/d.[20] Our results also differ from a large case-control study in Costa Rica, which supported an inverse association of ALA intake with nonfatal MI, with an odds ratio (95% CI) of 0.61 (0.42-0.80) for the top (2.4 g/d) vs. the bottom (1.1 g/d) decile of ALA intake.[13] Similar, but stronger, associations were observed for ALA status in adipose tissue in the same study. No further risk reductions were obtained beyond the 7th decile of ALA status, corresponding to an ALA intake of 1.8 g/d.[13] Although this retrospective study suggested that benefits of ALA on CHD could already be achieved at modest levels,[13,37] our prospective study did not support this. Misclassification of ALA intake, especially within a relatively narrow range of intake, may

have attenuated our associations. ALA intake levels in the Netherlands are comparable to most West European countries and the United States of America.[38] Within the range of ALA intake that we studied, the associations with CHD and stroke can therefore be extrapolated to other western populations.

Despite the small range of intake in our study, we did find an inverse association of ALA intake with incident stroke, which was most pronounced for ALA from salad dressings. It is not likely that ALA from different food sources would act differently. Although we adjusted for many potential confounders, including the intake of raw vegetables, the associations may still be influenced by a healthier diet and lifestyle of those who regularly eat raw vegetables with salad dressings. In general, correlated fatty acids in foods and residual confounding play an important role in cohort studies and results should therefore be judged with caution. Epidemiological studies of ALA intake or status and stroke are scanty. Our results are in line with a nested case-control study in middle-aged American men at high risk for CVD.[25] In that study, one SD increase of ALA in serum cholesteryl esters was associated with a 37% decrease in risk of stroke.

Humans can convert ALA into the longer-chain fatty acid EPA and eventually DHA, although conversion occurs to a limited extent.[39] Apart from potential indirect effects of ALA on CVD via conversion into EPA and DHA, ALA has been suggested to have anti-inflammatory,[6,11] anti-arrhythmic,[12] anti-thrombotic,[12,13] or neuroprotective effects.[14] However, others concluded that there was insufficient evidence that ALA influences risk factors for CVD.[15,16] Although CHD and ischemic stroke are both atherosclerotic disorders that have risk factors in common, we found differential associations of ALA intake for CHD and stroke. A proposed mechanism from animals studies for a protective effect of ALA on incident ischemic stroke is that ALA would be neuroprotective after induced ischemia, by beneficially affecting the brain blood flow.[14]

Concluding, in this generally healthy Dutch population, ALA intake was not associated with incident CHD. However, the data suggested that a low intake of ALA may be a risk factor for incident stroke, although more prospective studies are needed before definite conclusions can be drawn.

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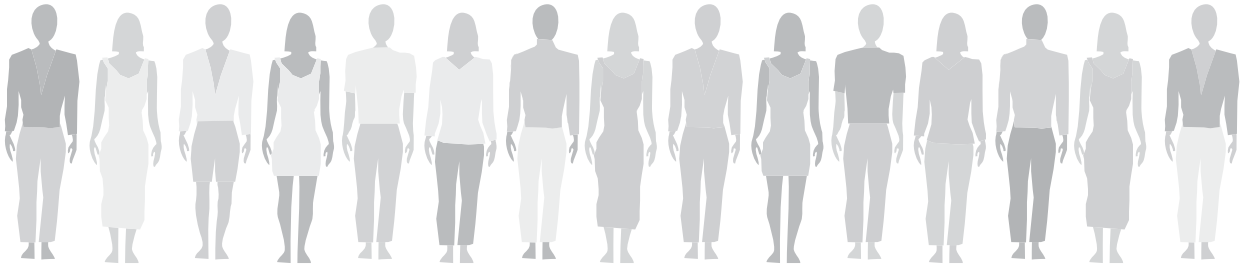

5

Marine n-3 fatty acids, fish consumption, and the 10-year risk of fatal and nonfatal coronary heart disease in a large population of Dutch adults with low fish intake

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Abstract

We assessed the dose-response relations within a low range of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and fish intake on fatal coronary heart disease (CHD) and nonfatal myocardial infarction (MI). In a Dutch population-based cohort study, EPA-DHA and fish intake were assessed at baseline among 21,342 participants aged 20-65 y with no history of MI or stroke. Hazard ratios were calculated with Cox proportional-hazard models. During 9-14 y of follow-up (mean 11.3 y), 647 participants (3%) died, of which 82 of CHD. Fatal CHD mainly comprised MI (64 cases). In total, 252 participants survived an MI. Median intakes in quartiles of EPA-DHA were 40, 84, 151, and 234 mg/d. Medians of fish consumption in quartiles were 1.1, 4.2, 10.7, and 17.3 g/d. Compared with the lowest quartile of EPA-DHA, participants in the top quartile had a 49% lower risk of fatal CHD (95% CI: 6-73%) and a 62% lower risk of fatal MI (95% CI: 23-81%). We observed inverse dose-response relations for EPA-DHA intake and fatal CHD (*P*-trend = 0.05) and fatal MI (*P*-trend = 0.01). Results were similar for fish consumption. Nonfatal MI was not associated with EPA-DHA or fish intake. In conclusion, in populations with a low fish consumption, EPA-DHA and fish may lower fatal CHD and MI risk in a dose-responsive manner. Low intakes of EPA-DHA or fish do not seem to protect against nonfatal MI.

Introduction

In 1985, Kromhout *et al.*[1] showed that a small amount of fish in the diet was associated with a lower risk of coronary heart disease (CHD) mortality in the Zutphen Study of 852 elderly Dutch men. In a meta-analysis of prospective cohort studies, He *et al.*[2] estimated that eating fish once per week was associated with a 15% lower risk of coronary death compared with a fish intake of less than once per month. Each 20-g/d increase in fish consumption was related to a 7% lower risk of CHD mortality (*P*-trend = 0.03). The marine-derived, very-long chain (n-3) PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are assumed to be primarily responsible for these health effects of fish. The meta-analysis of He *et al.*[2] also showed that the evidence for an inverse association of fish intake and risk of nonfatal myocardial infarction (MI) was weak (*P*-trend = 0.40), even though there was a significant inverse association of eating fish ≥5 times/wk compared with less than once per month.

Several randomized controlled trials (RCT) on fish and fish oil in relation to coronary and all-cause mortality have been conducted in cardiac patients. In the context of this paper, the findings in patients with a low level of fish consumption are most relevant. The first RCT using low levels of fatty fish or fish oil capsules as interventions showed significant reductions in fatal CHD[3,4] and sudden death.[4] Recent meta-analyses of RCT showed that fish oil supplementation significantly reduced fatal CHD[5] and fatal MI[6] in coronary patients.

Mozaffarian and Rimm[7] combined data from prospective cohort studies and RCT and estimated that a reduction of CHD mortality may be achieved with relatively low intakes of EPA and DHA. Modest consumption of fish (1-2 servings/wk, which is ~100-200 g fish/wk) was associated with a 36% lower risk of coronary death. They suggested that for the general population an intake of 250 mg/d of EPA-DHA (1 serving of fatty fish/wk) would be sufficient. Others have recommended target intakes of ~500 mg/d.[8-11] Most studies have mainly focused on fish consumption[12] as the main source of EPA and DHA. However, other foods like meat and eggs also contribute to the intake of these fatty acids[13] and may be important to take into account.

In this prospective cohort study, we investigated the dose-response relations of habitual intake of EPA-DHA and fish on fatal CHD and fatal and nonfatal MI within the low range of fish intake in The Netherlands.

Methods

Design and study population

The Monitoring Project on Risk Factors for Chronic Diseases (MORGEN) study is a Dutch population-based cohort of 22,654 men and women, aged 20-65 y. The MORGEN study contributes to the Dutch part of the European Prospective Investigation into Cancer and

Nutrition.[14] In the MORGEN study, information on diet, lifestyle, and cardiovascular risk factors was collected in 1993-1997. For the MORGEN study, random samples (stratified by gender and 5-y age groups) from civil registries of Amsterdam, Doetinchem, and Maastricht were drawn, representing three geographical parts of The Netherlands. The mean response rate was 45%. The study complies with the Declaration of Helsinki and the protocol was approved by the Medical Ethics Committee of the TNO Prevention and Health (Leiden, The Netherlands).

For the current analyses, we excluded participants who did not provide informed consent for vital status follow-up (n=701). We also excluded participants without dietary information (n=72) and 97 participants with extreme intakes of energy (<2,094 or >18,844 kJ for women and <3,350 and >20,938 kJ for men). Furthermore, participants with a history of MI or stroke at baseline (n=442) based on self-report or hospital admission data were excluded, resulting in 21,342 participants for the current analyses.

Dietary assessment

Dietary information was assessed at baseline with a self-administered 178-item FFQ.[15] The questionnaire included foods that covered the daily intake of each nutrient or food of interest for at least 90% of the population mean intake, based on the Dutch National Food Consumption Survey of 1987-1988. Participants were asked to report the usual frequency of consumption of the food items during the past year and their mean portion sizes. Participants indicated their answers in times per day, per week, per month, per year, or as never. For 28 food items, color photographs were used to estimate portion sizes. Information on habitual fish intake was obtained by questions on the absolute frequency of fish consumption combined with questions on the following types of fish: 1) lean and moderately fatty fish, including plaice, cod, fried fish, fish fingers; 2) fatty fish, including eel, mackerel, herring; 3) shrimps and mussels. Trained research assistants obtained information on unclear or missing items. After checking for improbable and inconsistent answers, the dietary data were converted into daily food and nutrient intakes and frequencies of food items by using the digital update (of 1998) of the Dutch food composition database (NEVO) of 1996.[16] The daily intakes of fatty acids were calculated with additional NEVO information of the 2001 release.[17]

The relative validity (intake assessed by the FFQ compared with intakes assessed by 12 monthly 24-h recalls) and reproducibility (measured by 2 repeated measurements) of the FFQ for food groups and some nutrients were assessed among 121 Dutch men and women.[15,18] The Spearman rank correlations for the reproducibility of the FFQ after 6 mo for fish intake were 0.49 for men and 0.61 for women. The relative validity (Spearman rank correlation) of the FFQ for fish intake was 0.32 for men and 0.37 for women (all $P<0.05$).

Mortality and morbidity data

After enrolment in the MORGEN study, the participants were followed for the occurrence of fatal CHD and fatal or nonfatal MI by linkage with several registries, including Statistics Netherlands for cause-specific mortality and the hospital discharge register (HDR; in Dutch: Landelijke Medische Registratie) for hospital admissions.[19]

Information on mortality follow-up was available from baseline until January 2007. CHD mortality was defined as International Classification of Diseases (ICD) 10[20] codes I20-I25 or ICD9[21] codes 410-414 and MI as ICD-10 codes I21-I22 or ICD-9 code 410 based on primary or secondary causes of death. For the analyses on fatal CHD and fatal MI, participants were followed until death, emigration, or they were censored at January 1, 2007. Deceased participants who had not given permission to obtain data on cause-specific death (n=43) were censored at date of death. Causes of death could not be obtained for participants who died outside The Netherlands.

Information on nonfatal MI (defined as ICD-9 code 410) was based on hospital admission data. These data were available from baseline until January 2006. Hospital admissions followed by death at the same date were regarded as fatal events. Participants with both information on vital status and hospital admissions (n=20,880) were followed until the first nonfatal MI event or they were censored at death, emigration, or January 1, 2006, whichever occurred first. In The Netherlands, hospital admissions are coded by gender, date of birth, and the numeric part of the postal code. At least 88% of the hospital admissions of our cohort could be uniquely linked to a participant in our cohort.[19]

Assessment of covariates

Body weight, height, and blood pressure were measured by trained research nurses at a municipal health service site.[22] A self-administered questionnaire was used to assess the presence of known diabetes, history of MI and stroke, medication use, vitamin or mineral supplement use (yes/no), family history of MI, educational level, alcohol consumption, and cigarette smoking. Physical activity was assessed only during baseline measurements in 1994-1997, which comprised 77% of our cohort.[23] For this subset, we calculated whether participants complied with being physically active during 30 min with a moderate intensity on 5 d/wk.

Participants donated blood (nonfasting) in which the levels of total serum cholesterol and serum HDL cholesterol were assessed at the Lipid Reference Laboratory of the Erasmus Medical Center in Rotterdam, using enzymatic methods.[24] Blood pressure was measured, with the participant in a sitting position. Systolic pressure was recorded at the appearance of sounds (first-phase Korotkoff) and diastolic blood pressure was recorded at the disappearance of sounds (fifth-phase Korotkoff). Blood pressure was measured twice, after the first measurement heart rate was counted for 30 s, followed by the second measurement.[25] The mean of the 2 measurements was used in the analyses.

Statistical analysis

We used Cox proportional-hazard models with follow-up time as time metric to estimate relative risks of fatal CHD, fatal MI, and nonfatal MI in quartiles of habitual intake of EPA-DHA and total fish. We calculated hazard ratios with 95% CI with the lowest quartiles of EPA-DHA and fish intake as the reference category. Our models fulfilled the proportional-hazards assumption. Participants' characteristics in quartiles of EPA-DHA intake are presented as mean ± SD, median [interquartile range (Q1-Q3)], or percentages, unless otherwise noted. The correlation between the intake of EPA-DHA and total fish was assessed with the Spearman rank correlation test.

In addition to an age- and gender-adjusted model (model 1), we used multivariable-adjusted models (model 2) that included total energy intake (kJ/d), BMI (kg/m^2), alcohol intake (based on the calculation of total ethanol intake in g/d by FFQ), cigarette smoking (never, former, current), socioeconomic status (primary school, secondary school, up to higher vocational training, completed higher vocational training or university), vitamin or mineral supplement use (yes/no), use of drugs for hypertension or hypercholesterolemia (yes/no), family history (yes/no) of CHD (MI of father before age of 55 y or MI of mother before age of 65 y), fruit consumption (g/d), vegetable consumption (g/d), saturated fat intake (g/d). Covariates were selected based on what we know from the literature to be important confounders of the relation between (n-3) PUFA and CHD. Stratified analyses did not provide evidence for interaction by gender or age of the association of EPA-DHA or fish intake with different outcomes. Therefore, we combined men and women and different age groups. Possible confounding by physical activity was checked in the subgroup of participants with information on physical activity (n=16,421). Analyses were repeated for quartiles of EPA-DHA from marine sources only. All probability values are 2-tailed with α=0.05 (SAS/STAT software, version 9.1; SAS Institute).

Results

Population characteristics

Participants were 42.1 ± 11.2 y old at baseline and 45% were male. Median intakes of EPA-DHA and fish were 114 (62-195) mg/d and 7.4 (3.3-14.0) g/d, respectively. About 40% of the participants consumed fish less than once per month and 8.5% reported eating no fish at all. The median frequency of fish consumption was 2 (1-4) times/mo. The consumption of lean fish + moderately fatty fish, which were combined in our questionnaire, was 3-4 times as high as the consumption of fatty fish. This resulted in a higher absolute intake of EPA-DHA from lean + moderately fatty fish than from fatty fish (data not shown).

During 9-14 y of follow-up (mean 11.3 y), 647 (3%) participants died of which 82 died of CHD. Fatal CHD mainly comprised MI (64 cases). In total, 252 participants survived an MI.

Table 5.1 Baseline characteristics of 21,342 Dutch adults, aged 20-65 y, by quartiles of EPA-DHA intake[a]

	Quartiles of EPA-DHA intake, *mg/d* (*range*)			
	1 (<62)	2 (62-113)	3 (114-194)	4 (>194)
n	5,336	5,335	5,335	5,336
Median EPA-DHA intake, mg/d	40	84	151	234
Male gender, %	39	45	45	51
Age, y	41.1 ± 11.8	41.8 ± 11.2	42.4 ± 10.8	43.3 ± 10.8
Body mass index, kg/m^2	24.8 ± 4.0	25.0 ± 3.9	25.0 ± 3.9	25.2 ± 4.0
Fish consumers (≥1 servings/mo),[b] %	<1	40	98	100
Fish intake, g/d	1.4 ± 1.4	4.9 ± 2.2	10.9 ± 3.7	22.0 ± 14.3
EPA, mg/d	10 ± 6	25 ± 7	48 ± 11	98 ± 60
DHA, mg/d	28 ± 11	61 ± 11	105 ± 18	197 ± 106
EPA-DHA, mg/d	39 ± 15	86 ± 16	152 ± 25	295 ± 163
EPA-DHA from fish, mg/d	13 ± 13	53 ± 21	117 ± 33	255 ± 164
PUFA, % of energy intake	6.6 ± 1.7	6.8 ± 1.6	6.8 ± 1.7	7.0 ± 1.7
SFA, % of energy intake	14.6 ± 2.7	14.6 ± 2.4	14.3 ± 2.5	14.1 ± 2.6
Total fat, % of energy intake	34.7 ± 5.1	35.3 ± 4.8	34.9 ± 5.0	34.9 ± 5.1
Energy intake, MJ/d	8.9 ± 2.7	9.5 ± 2.7	9.6 ± 2.7	9.8 ± 2.9
Smoking, %				
Never	37	34	34	33
Former	28	30	31	29
Current	35	35	36	38
Alcohol consumption,[c] g/d	3.1 (0.3-11.8)	5.8 (1.1-16.1)	7.5 (1.5-19.5)	8.7 (1.5-22.5)
Highly educated,[d] %	19	22	28	27
Dutch ethnicity, %	98	97	97	94
Physically active,[e] %	63	67	67	67
Parental history of MI, %	10	9	9	10
Self-reported diabetes mellitus, %	1.0	0.8	1.0	1.7
Serum total cholesterol,[f] mmol/L	5.3 ± 1.0	5.2 ± 1.0	5.3 ± 1.1	5.3 ± 1.1
Serum HDL-cholesterol,[f] mmol/L	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4
Systolic blood pressure, mm Hg	120.4 ± 15.9	120.4 ± 15.9	120.8 ± 16.1	121.7 ± 16.6
Diastolic blood pressure, mm Hg	76.5 ± 10.4	76.6 ± 10.5	76.6 ± 10.7	76.9 ± 10.9
Use of cholesterol-lowering drugs, %	1.0	0.7	0.9	1.3
Use of antihypertensive drugs, %	4.5	4.0	4.7	4.8
Supplement use,[g] %	28	29	33	34

[a] Values are means ± SD, unless indicated otherwise.
[b] 1 serving = ~100 g.
[c] Median with interquartile range.
[d] University or higher vocation training.
[e] Compliant to being physically active during 30 min with a moderate intensity on 5 d/wk. Available in subsample of participants enrolled between 1994 and 1997 (n=16,421).
[f] Nonfasting.
[g] Vitamin or mineral supplement.

Median EPA-DHA intakes in quartiles were 40, 84, 151, and 234 mg/d. The ratio of EPA:DHA was ~1:2. The main source of EPA-DHA was fish (63%). In all quartiles, the amount of EPA-DHA from other sources than fish was ~30 mg/d. In the higher quartiles, participants were slightly older, more likely to be male, and higher educated. In the lowest quartile, 63% of the participants complied with the Dutch guideline for physical activity. In the other 3 quartiles 67% complied. Higher intakes of EPA-DHA were associated with a higher intake of energy and alcohol (**Table 5.1**). The Spearman rank correlation between total EPA-DHA intake and fish consumption was 0.95.

EPA-DHA intake, fish consumption, and CHD

After adjustment for potential confounders, the risk of fatal CHD was inversely associated with EPA-DHA intake, with a 49% lower risk (95% CI: 6%-73%) in the top quartile of EPA-DHA compared with the reference group. We found a stronger association between fatal MI and EPA-DHA intake, with a 62% lower risk in the top quartile. A dose-response relation was found for both fatal CHD (*P*-trend = 0.05) and fatal MI (*P*-trend = 0.01). EPA-DHA intake was not associated with nonfatal MI (**Table 5.2**). We repeated our analyses for quartiles of EPA-DHA from marine sources only. These results did not differ from the results on total EPA-DHA (data not shown).

Table 5.2 Associations of fatal CHD and (non)fatal MI by quartiles of EPA-DHA intake in 21,342 Dutch men and women[a]

	Quartiles of EPA-DHA intake, *mg/d, (range)*				
	1 (<62)	2 (62-113)	3 (114-194)	4 (>194)	*P*- trend
n	5,336	5,335	5,335	5,336	
Median EPA-DHA, mg/d	40	84	151	234	
Fatal CHD					
Events, n	24	18	20	20	
Model 1[b,c]	1.0 (ref)	0.74 (0.40-1.36)	0.76 (0.42-1.37)	0.68 (0.38-1.23)	*0.27*
Model 2[d,e]	1.0 (ref)	0.68 (0.36-1.25)	0.65 (0.36-1.19)	0.51 (0.27-0.94)	*0.05*
Fatal MI					
Events, n	21	13	16	14	
Model 1[b,c]	1.0 (ref)	0.60 (0.30-1.20)	0.69 (0.36-1.31)	0.54 (0.27-1.06)	*0.13*
Model 2[d,e]	1.0 (ref)	0.57 (0.28-1.14)	0.56 (0.29-1.09)	0.38 (0.19-0.77)	*0.01*
Nonfatal MI					
Events, n	57	61	61	73	
Model 1[b,f]	1.0 (ref)	1.06 (0.74-1.52)	0.99 (0.69-1.42)	1.10 (0.78-1.56)	*0.10*
Model 2[d,g]	1.0 (ref)	1.07 (0.74-1.55)	1.04 (0.72-1.50)	1.07 (0.74-1.54)	*0.18*

[a] Values are hazard ratios (95% CI), with the first quartile as the reference category.
[b] Model 1: adjusted for age and gender.
[c] n=21,342.
[d] Model 2: model 1 with additional adjustments for BMI, total energy intake, ethanol intake, cigarette smoking, social economic status, vitamin or mineral supplement use, use of drugs for hypertension or hypercholesterolemia, parental history of myocardial infarction, SFA, fruit, and vegetables.
[e] n=21,055.
[f] n=20,880.
[g] n=20,605.

Median intakes in quartiles of fish consumption were 1.1, 4.2, 10.7, and 17.3 g/d. Like for EPA-DHA, consuming more fish was associated with a lower risk of fatal CHD and fatal MI. Similar to our results on EPA-DHA intake, the associations were dose-dependent. Fish consumption was not associated with nonfatal MI (**Table 5.3**). We have additionally included monounsaturated fatty acids, linoleic acid, and α-linolenic acid in our multivariable models. However, this yielded similar results for both our analyses on total EPA-DHA and fish consumption. Our population consisted of only 1% of diabetic patients and our results did not change when we excluded diabetic patients (data not shown).

Discussion

In our healthy Dutch population with a low habitual fish intake, EPA-DHA and fish consumption were inversely associated with fatal CHD and fatal MI, but not with nonfatal MI. The risk of fatal CHD in the highest quartile of EPA-DHA intake (~250 mg/d) was ~50% lower compared with

Table 5.3 Associations of fatal CHD and (non)fatal MI by quartiles of fish intake in 21,342 Dutch men and women[a]

	Quartiles of fish intake, *g/d (range)*				
	1 (<3.3)	2 (3.3-7.3)	3 (7.4-14.0)	4 (>14)	*P*-trend
n	5,284	5,401	5,258	5,399	
Median fish intake, g/d	1.1	4.2	10.7	17.3	
Median EPA:DHA, mg/d	39	82	148	228	
Median EPA:DHA from fish, mg/d	11	46	119	191	
Fatal CHD					
Events, n	25	24	14	19	
Model 1[b,c]	1.0 (ref)	0.95 (0.54-1.66)	0.52 (0.27-0.99)	0.63 (0.35-1.15)	*0.06*
Model 2[d,e]	1.0 (ref)	0.92 (0.52-1.61)	0.50 (0.26-0.97)	0.52 (0.28-0.95)	*0.02*
Fatal MI					
Events, n	19	22	11	12	
Model 1[b,c]	1.0 (ref)	1.13 (0.61-2.10)	0.53 (0.25-1.11)	0.53 (0.25-1.09)	*0.02*
Model 2[d,e]	1.0 (ref)	1.12 (0.60-2.08)	0.50 (0.24-1.06)	0.40 (0.19-0.86)	*<0.01*
Nonfatal MI					
Events, n	61	57	66	68	
Model 1[b,f]	1.0 (ref)	0.92 (0.64-1.32)	1.02 (0.72-1.44)	0.96 (0.68-1.36)	*0.15*
Model 2[d,g]	1.0 (ref)	0.96 (0.67-1.39)	1.07 (0.75-1.54)	1.01 (0.71-1.45)	*0.14*

[a] Values are hazard ratios (95% CI), with the first quartile as the reference category.
[b] Model 1: adjusted for age and gender.
[c] n=21,342.
[d] Model 2: model 1 with additional adjustments for BMI, total energy intake, ethanol intake, cigarette smoking, social economic status, vitamin or mineral supplement use, use of drugs for hypertension or hypercholesterolemia, parental history of myocardial infarction, SFA, fruit, and vegetables.
[e] n=21,055.
[f] n=20,880.
[g] n=20,605.

the lower quartile (~40 mg/d). Within this low range of intake, the inverse associations with fatal CHD and fatal MI risk were graded. Similar results were found for fish, i.e. participants who consumed only 17 g/d (~1 portion of fish/wk) had an ~50% lower risk of fatal CHD.

This study has several strengths, including complete information on vital status with little loss to follow-up of a large population-based cohort and detailed information on potential confounders. Both fatal and nonfatal CHD could be studied in relation to fish and EPA-DHA. An extensive FFQ was used that allowed calculation of EPA-DHA from the whole diet. However, there were also limitations. First, data on physical activity, which could be an important confounder, was available for only 77% of the participants. We performed multivariable analyses with and without adjustment for physical activity in this subgroup, which yielded similar risk estimates for EPA-DHA and fish intake in relation to CHD. We therefore think that residual confounding by physical activity is not a major issue in the present study. Second, misclassification of participants for EPA-DHA and fish intake may have occurred. The relative validity of our FFQ for fish intake was only 0.32 for men and 0.37 for women.[15] Because we excluded participants with a history of MI or stroke, we expect misclassification at baseline to be random rather than dependent on disease outcome. Random misclassification could have attenuated the risk estimates in the present study. Third, we obtained data on nonfatal MI via linkage with the national HDR. Eighty-eight percent of the hospital admissions can be uniquely linked to an individual on basis of gender, date of birth, and postal code.[19] In a validation study, the HDR was compared with the detailed clinical registry of cardiovascular patients of the Cardiology Department of the Maastricht University Hospital, showing a relatively high sensitivity (84%) and positive predictive value (97%) for MI.[26] The region of Maastricht is 1 of the 3 regions of the MORGEN study. Should nonfatal MI cases in our study be missed by this procedure, this is unlikely to be related to the fish intake and will therefore not have biased our results.

With respect to fatal CHD, our results are comparable to the Zutphen Study, in which the consumption of 1-2 fish meals/wk was associated with half the risk of CHD mortality compared with lower intakes.[1] In a pooled analysis of prospective cohort studies and clinical trials, Mozaffarian and Rimm[7] estimated that a daily intake of 250 mg of EPA-DHA (1-2 servings of fish/wk) was associated with a 36% lower risk of fatal CHD with little additional benefit above 250 mg/d. The range of EPA-DHA intake in our study was mostly below 250 mg/d and we found risk reductions up to 49% for fatal CHD. This is also larger than observed in the meta-analysis by He *et al*.,[2] who found a 15% lower risk of fatal CHD for weekly fish consumption. The dose-response relation of He *et al*.[2] might be attenuated by studies with much higher intakes of fish with little extra benefit. Another reason for our stronger associations could be that our relatively young cohort had a lower baseline risk to develop fatal CHD compared with the cohorts in the above-mentioned meta-analysis, which may have inflated our risk estimates to some extent. In a recent meta-analysis of fish oil supplementation trials, a 20% reduced risk of cardiac death was found compared with placebo.[5] Fish oil doses in these trials amounted to several grams per day, which cannot be achieved through diet alone. Furthermore, the Japanese JELIS trial[27] in

18,645 hypercholesterolemic patients largely influenced the overall risk estimates of this meta-analysis. In this trial, on top of a high habitual fish intake, no effect of 5-y supplementation with 1.8 g/d EPA on fatal CHD was found.

We found no associations between nonfatal MI and low levels of EPA-DHA or fish intake. These findings are concordant with the meta-analysis of prospective cohort studies of He *et al*.[2] in which a significant inverse association for fish intake and nonfatal CHD was found only for eating fish ≥5 times/wk compared with less than once per month, which is much higher than the intake in our cohort. In addition, Japanese studies showed that at high levels of intake, fish and EPA-DHA may be protective against nonfatal CHD. In the Japan Public Health Center-Based Study, the relative risk for nonfatal MI was 0.43 (95% CI: 0.23-0.81) in participants with a median fish intake of 180 g/d, compared with participants with a daily intake of 23 g/d.[28] In the above-mentioned JELIS trial, the risk of nonfatal CHD was reduced by 19%.[27] However, the Japanese habitual intake of fish (mean of 85 g/d) is much higher than the Dutch diet. Currently, no data are available for RCT with low levels of EPA and DHA intake in relation to CHD incidence and mortality.

In the present cohort with a low range of fish consumption, EPA-DHA intake was inversely related to CHD mortality and even stronger to fatal MI. In various studies, low doses of EPA and DHA are associated with a lower risk of fatal CHD but not nonfatal CHD.[2] A hypothesis for this differential effect is that EPA and DHA could prevent fatal cardiac arrhythmia.[9,29-31] Life-threatening cardiac arrhythmias are major contributors to fatal CHD. These arrhythmias are less likely to occur in the case of a less-severe MI with less cardiac tissue damage. Although in recent RCT, marine (n-3) PUFA did not protect against arrhythmia in ICD patients,[32] the arrhythmia hypothesis is still the major hypothesis in CHD primary prevention studies.[33] Regrettably, we were not able to examine sudden cardiac death as a separate outcome in the present study because of the limited number of cases.

We conclude that in a population with low levels of fish consumption, higher intakes of EPA-DHA and fish may protect against fatal CHD in a dose-responsive manner. Intake of only a small amount of fish may be beneficial to cardiac health, although no protection against nonfatal MI may be expected.

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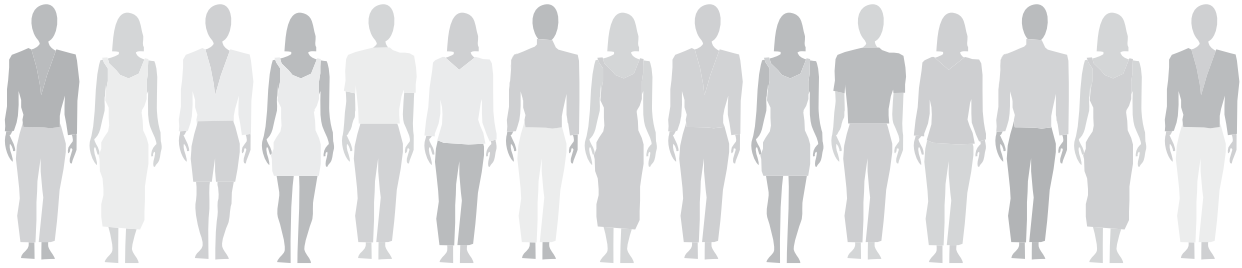

6

Gender-specific associations of marine n-3 fatty acids and fish consumption with 10-year incidence of stroke

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Abstract

Background: There is some evidence that the association of fish and fish fatty acids with stroke risk differs between men and women. We investigated the gender-specific associations of habitual intake of the fish fatty acids eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA) and fish on incident stroke in a population-based study in the Netherlands.

Methods: We prospectively followed 20,069 men and women, aged 20-65 years, without cardiovascular diseases at baseline. Habitual diet was assessed with a validated 178-item food frequency questionnaire. Incidence of stroke was assessed through linkage with mortality and morbidity registers. Cox proportional hazards models were used to estimate multivariable-adjusted hazard ratios (HR) and 95% confidence intervals (95% CI).

Results: During 8-13 years of follow-up, 221 strokes occurred. In women, an inverse dose-response relation (*P*-trend = 0.02) was observed between EPA-DHA intake and incident stroke, with an HR of 0.49 (95% CI: 0.27-0.91) in the top quartile of EPA-DHA (median 225 mg/d) as compared to the bottom quartile (median 36 mg/d). In men, the HR (95%CI) for the top quartile of EPA-DHA intake was 0.87 (0.51-1.48) (*P*-trend = 0.36). Similar results were observed for fish consumption and stroke incidence.

Conclusion: A higher EPA-DHA and fish intake is related to a lower stroke risk in women, while for men an inverse association could not be demonstrated.

Introduction

Worldwide, stroke is the second largest cause of mortality and a major cause of long-term disability.[1,2] As part of a healthy diet, fish consumption is advised to reduce the risk of cardiovascular diseases.[3-5] Although literature strongly suggests that consuming fish protects against coronary heart disease,[6] data for protection against stroke are less convincing.

Several,[7-13] although not all,[14-18] prospective cohort studies showed inverse associations of fish consumption with stroke. In a meta-analysis, He *et al*. summarized prospective cohort studies published through 2003 and concluded that fish consumption once a week compared to less than once per month was related to a 13% (HR: 0.87; 95% CI: 0.77-0.98) lower stroke risk.[19] In three cohort studies[9-11] with information on types of stroke, consuming fish more than once a month was associated with a 30-35% lower risk of ischemic stroke, and not to hemorrhagic stroke.[19]

In four[8,9,12,13] out of six[8,9,12,13,17,20] prospective studies carried out in women from western countries, fish consumption was inversely associated with stroke, whereas only two[7,10] out of seven[7,8,10,12,14,15,20] studies reported inverse associations in men. In addition, fish intake was more strongly inversely related to stroke in women than in men in two cohort studies that stratified by gender.[8,12] Less data are available for the intake of the fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish, but those available were in agreement with those on fish consumption and stroke incidence.[9,10,14,16,18]

We investigated the gender-specific associations of habitual intake of EPA-DHA and fish with 10-year incidence of stroke in a large population-based study in the Netherlands.

Methods

Ethical statement

This research was performed in accordance with the ethical principles for medical research involving human subjects outlined in the Declaration of Helsinki. This research was approved by the Medical Ethics Committee of TNO Prevention and Health (Leiden, The Netherlands). All participants gave written informed consent.

Design and study population

The "Monitoring Project on Chronic Disease Risk Factors" (MORGEN) study is a population-based cohort of 22,654 men and women, aged 20-65 years in the Netherlands. The MORGEN study contributes to the Dutch part of the European Prospective Investigation into Cancer and Nutrition (EPIC).[21] Baseline (1993-1997) information on diet, lifestyle, and cardiovascular risk factors was collected and participants were followed for cardiovascular endpoints. The study

complies with the Declaration of Helsinki and the protocol was approved by the Medical Ethics Committee of the TNO Prevention and Health Institute (Leiden, The Netherlands). Written informed consent was obtained from each participant.

Participants who did not provide informed consent for vital status follow-up were excluded as well as participants with no dietary information or with extreme energy intakes (<500 or >4,500 kcal for women and <800 or >5,000 kcal for men). Furthermore, we excluded participants with a history of myocardial infarction or stroke at baseline, participants with self-reported diabetes and participants who used serum lipid modifying agents or antihypertensive drugs, resulting in 20,069 participants (8,988 men and 11,081 women) for the present analysis.

Dietary assessment

Habitual diet was assessed at baseline with a self-administered 178-item Food Frequency Questionnaire (FFQ), covering the previous year.[22,23] Participants indicated consumption of main food groups in times per day, per week, per month, or as never, combined with questions on the relative intake of foods within food groups (seldom/never, sometimes, often, mostly/always). Information on habitual fish intake was obtained by questions on the absolute frequency of fish consumption combined with questions on the following types of fish: 1. lean and moderately fatty fish, including plaice, cod, fried fish, fish fingers; 2. fatty fish, including eel, mackerel, herring; 3. shrimps and mussels. Nutrient intakes were calculated with the "Dutch food composition database" of 1998. For individual fatty acids, we used the updated table of 2001.

The relative validity of the FFQ against 12 monthly 24-h recalls and reproducibility of the FFQ after 6 months for food groups and nutrients were assessed among 121 Dutch men and women.[22,23] The Spearman rank correlations for the reproducibility of the FFQ after 6 months for fish intake were 0.49 for men and 0.61 for women. The relative validity (Spearman correlation) of the FFQ for fish intake was 0.32 for men and 0.37 for women.[23] The rank correlations for dietary EPA and DHA (mg/d) with cholesteryl ester plasma levels in a subset of the current population were in men (n=268) 0.34 for EPA and 0.47 for DHA and in women (n=189) 0.36 for EPA and 0.33 for DHA (unpublished results).

Case ascertainment and follow-up

Vital status was checked through linkage with the municipal population registers. For those who died, information on the cause of death was obtained from Statistics Netherlands. Information on nonfatal stroke was provided by the national hospital discharge register based on a validated probabilistic method described in more detail elsewhere.[24] On the national level, data from the Dutch hospital discharge register can be uniquely matched to a single person for at least 88% of the hospital admissions.[24] Incident total stroke comprised fatal and nonfatal stroke, corresponding with International Classification of Diseases (ICD-10, WHO) codes I60-I66 and

G45. This definition also included transient ischemic attacks (TIA) (G45). Ischemic stroke included I63, I65, I66, and G45, and hemorrhagic stroke included I60-I62. For hospital admissions and for causes of death coded until January 1, 1996, corresponding ICD9 codes were used. If the dates of hospital admission and death coincided, the event was considered fatal.

Data collection on risk factors

The baseline measurements were previously described in detail by Verschuren *et al*.[25] Body weight, height, and blood pressure were measured by trained research nurses. Blood pressure was measured twice, with the subject in sitting position. The mean of the two measurements was used in the analyses. Nonfasting blood was analyzed for plasma total and high-density lipoprotein (HDL) cholesterol. A self-administered questionnaire was used to assess the presence of diabetes, history of myocardial infarction or stroke, medication use, parental history of premature myocardial infarction, education level, and cigarette smoking. Alcohol intake (based on the FFQ) was calculated in glasses/d and was categorized as no intake, low to moderate intake (men ≤2 and women ≤1 glasses/d), or high intake (men >2 and women >1 glasses/d). Baseline physical activity was assessed with a validated questionnaire in 76% of the cohort, enrolled between 1994-1997.[26] For this subset, we calculated whether participants were engaged in cycling (yes/no) and sports (yes/no), both activities with a metabolic equivalent score ≥4, which were significantly inversely related to cardiovascular disease incidence in this population.[27]

Statistical analysis

Follow-up time was calculated from date of enrollment until death, incident stroke, date of loss-to-follow-up due to emigration out of the Netherlands (n=693) or 1 January 2006, whichever occurred first. We used Cox proportional hazard models to estimate hazard ratios (HR) with 95% confidence intervals (95% CI) for the association of gender-specific quartiles of EPA-DHA and total fish intake with stroke incidence. The analyses were repeated for stroke subtypes, i.e. ischemic stroke and hemorrhagic stroke. The proportional hazards assumption was tested and not rejected based on Schoenfeld residuals and visual inspection. Participants' characteristics in quartiles of EPA-DHA intake are presented as mean±SD, median [interquartile range (Q1-Q3)], or percentages, depending on the type and distribution of variables. Interactions of EPA-DHA and fish intake with gender were statistically tested with the likelihood ratio test comparing the fully adjusted model of the total group (men and women combined) with a similar model with additional product terms of gender and quartiles of intake. The correlation between the intake of EPA-DHA and total fish was assessed with the Spearman rank correlation test.

In addition to an age adjusted model [model 1], we used multivariable-adjusted models [model 2] that included total energy intake (kcal/d), body mass index (kg/m^2), alcohol intake (none,

low to moderate, or high), cigarette smoking (never, former, current), educational level (primary school, secondary school, up to higher vocational training, completed higher vocational training or university), parental history of premature myocardial infarction (yes/no; for father <55 y and for mother <65 y), intake of dietary fiber (g/d), vitamin C (mg/d), beta-carotene (mg/d), saturated fatty acids (en%), *trans*-fatty acids (en%), monounsaturated fatty acids (en%), linoleic acid (en%), and alpha-linolenic acid (en%).

To examine whether systolic blood pressure could be an intermediate factor in the association of EPA-DHA or fish intake with stroke, we added this variable to the multivariable model and examined changes in HRs. Possible confounding by physical activity (cycling and sports) was checked in the subgroup of participants for which these data were available (n=15,423). All *P*-values are two-tailed with α=0.05. Statistical analyses were performed with Statistical Analysis Software (SAS), version 9.2.

Results

The median EPA-DHA intake across quartiles varied from 36 to 225 mg/d in women and from 44 to 241 mg/d in men. In the higher quartiles participants were slightly older, higher educated, and they consumed more energy and alcohol. In men, but not in women, EPA-DHA intake was positively associated with current smoking (**Table 6.1**). Eight per cent of the participants never consumed fish and 40% consumed fish less than once per month. The median fish consumption was twice per month. The Spearman rank correlation between total fish consumption and EPA-DHA intake was 0.95. We observed no interaction between EPA-DHA or fish intake and gender in relation to incident stroke (*P* for interaction = 0.30, both for EPA-DHA and fish).

During 8-13 years of follow-up (median 10.5 years), 221 incident strokes occurred. Total stroke comprised 142 ischemic strokes (including 60 TIAs), 47 hemorrhagic strokes, and 32 unspecified strokes. Rates for TIA were similar for men and women. Men had higher incidence rates of total and ischemic stroke (excluding TIA), whereas women had higher rates of hemorrhagic stroke (**Table 6.2**). Women were on average 5 years younger (~47y) than men (~52y) when their first stroke occurred.

After adjustment for potential confounders, we found an inverse dose-response relation (*P*-trend = 0.02) of EPA-DHA intake with incident total stroke in women. The HR (95% CI) for quartile 4 (median intake: 225 mg/d) was 0.49 (0.27-0.91) compared to the bottom quartile (median intake: 36 mg/d). EPA-DHA intake was not significantly associated with incident stroke in men. The HR for total stroke in quartile 4 was 0.87 (0.51-1.48) (**Table 6.3**). Results of fish consumption were similar to those of EPA-DHA intake. Women in the top quartile of fish consumption had a significantly lower risk of total stroke (HR: 0.51; 95% CI: 0.26-0.94) (*P*-trend = 0.01), while men in the top quartile had a HR of 0.75 (95% CI: 0.44-1.26) (**Table 6.4**).

Table 6.1 Baseline characteristics of 20,069 Dutch men and women, aged 20-65y, by quartiles of EPA-DHA intake

	Women				Men			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Range EPA-DHA (median), mg/d	<57 (36)	57-106 (77)	107-188 (142)	>188 (225)	<66 (44)	66-118 (89)	119-198 (157)	>198 (241)
n	2,770	2,770	2,771	2,770	2,247	2,247	2,247	2,247
Age, y	40.4 ± 11.9	40.3 ± 11.1	41.1 ± 10.8	42.5 ± 10.8	41.2 ± 11.4	41.2 ± 11.0	42.2 ± 10.6	43.2 ± 10.7
Fish, g/d	1.3 ± 1.3	4.7 ± 2.1	10.6 ± 3.5	22.0 ± 14.2	1.5 ± 1.4	5.0 ± 2.2	11.1 ± 3.8	21.9 ± 13.9
EPA, mg/d	10 ± 5	24 ± 7	46 ± 10	94 ± 58	11 ± 6	26 ± 7	49 ± 11	102 ± 63
DHA, mg/d	26 ± 10	56 ± 11	99 ± 18	189 ± 103	31 ± 12	65 ± 12	109 ± 18	202 ± 105
ALA, en%	0.55 ± 0.17	0.55 ± 0.15	0.56 ± 0.16	0.56 ± 0.15	0.53 ± 0.15	0.54 ± 0.15	0.53 ± 0.15	0.54 ± 0.15
Linoleic acid, en%	5.26 ± 1.60	5.36 ± 1.47	5.43 ± 1.49	5.53 ± 1.58	5.28 ± 1.60	5.39 ± 1.55	5.36 ± 1.56	5.46 ± 1.63
Saturated fatty acids, en%	14.7 ± 2.6	14.7 ± 2.4	14.4 ± 2.5	14.1 ± 2.6	14.4 ± 2.5	14.4 ± 2.4	14.2 ± 2.4	14.2 ± 2.5
Total fatty acids, en%	34.9 ± 5.2	35.5 ± 4.9	35.1 ± 5.0	34.9 ± 5.1	34.6 ± 5.0	35.1 ± 4.7	34.7 ± 5.0	35.0 ± 5.0
Total energy, MJ/d	7.9 ± 2.1	8.4 ± 2.1	8.5 ± 2.1	8.6 ± 2.3	10.6 ± 2.7	11.0 ± 2.8	11.0 ± 2.8	11.3 ± 2.9
Body mass index, kg/m^2	24.4 ± 4.1	24.5 ± 4.1	24.4 ± 4.1	24.7 ± 4.1	25.1 ± 3.6	25.3 ± 3.4	25.2 ± 3.3	25.4 ± 3.5
Smoking, %								
Never	39	36	35	36	34	31	31	28
Former	24	28	28	27	32	34	34	30
Current	37	36	37	37	34	35	35	42
Alcohol consumption, %								
None	23	16	15	16	10	7	7	8
Low to moderate	60	62	58	53	62	61	56	50
High	17	22	27	30	28	32	37	41
Highly educated,[a] %	17	21	26	26	24	26	32	29
Dutch ethnicity, %	98	98	97	95	98	96	96	94
Physical activity[b]								
Engaged in cycling, %	60	62	60	61	55	59	59	58
Engaged in sports, %	33	37	39	38	36	40	40	37
Parental history of premature MI, %	10	9	8	9	9	9	9	9
Serum total cholesterol,[c] mmol/l	5.2 ± 1.0	5.2 ± 1.0	5.2 ± 1.1	5.3 ± 1.0	5.2 ± 1.0	5.3 ± 1.1	5.3 ± 1.1	5.4 ± 1.1
Serum HDL-cholesterol,[c] mmol/l	1.5 ± 0.4	1.5 ± 0.4	1.5 ± 0.4	1.5 ± 0.4	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3
Systolic blood pressure, mm Hg	116.7 ± 15.5	116.2 ± 15.6	116.2 ± 14.9	117.1 ±15.8	123.7 ± 13.9	124.1 ± 14.6	123.9 ± 14.6	124.4 ± 15.5
Diastolic blood pressure, mm Hg	74.3 ± 9.9	74.5 ± 10.3	73.8 ± 9.9	74.2 ± 10.3	78.4 ± 9.9	78.5 ± 10.1	78.5 ± 10.1	78.4 ± 10.7

Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; HDL, High Density Lipoprotein; Q, quartiles.

[a] University or higher vocation training.

[b] Available for participants enrolled between 1994 and 1997 (n=15,423).

[c] Nonfasting.

Table 6.2 Incidence rates of total stroke and stroke subtypes in 20,069 Dutch men and women, aged 20-65y

	Women		Men	
	Incidence rate[a]	%	Incidence rate	%
Total stroke[b]	9.2		12.4	
Ischemic stroke excluding TIA[b]	2.6	28	5.6	45
TIA[b]	2.9	31	2.9	24
Hemorrhagic stroke[b]	2.7	29	1.7	14
Unspecified	1.0	11	2.2	17

Abbreviation: TIA, transient ischemic attack.

[a] Incidence rates per 10,000 person years.

[b] International Classification of Diseases (ICD-10) codes were I60-I66 and G45 for total stroke; I63, I65, I66, for ischemic stroke excluding TIA, G45 for TIA, and I60-I62 for hemorrhagic stroke.

Table 6.3 Associations of incident stroke by quartiles of EPA-DHA intake in 20,069 Dutch men and women[a]

	Total stroke[b]			Ischemic stroke		Hemorrhagic stroke	
Intake, mg/d Range (median)	Cases	Model 1[c]	Model 2[d]	Cases	Model 2[d]	Cases	Model 2[d]
Women							
Q1: <57 (36)	33	1.0 (ref)	1.0 (ref)	19	1.0 (ref)	9	1.0 (ref)
Q2: 57-106 (77)	28	0.88 (0.53-1.45)	0.89 (0.53-1.49)	17	0.98 (0.50-1.91)	7	0.73 (0.27-2.00)
Q3: 107-188 (142)	28	0.86 (0.52-1.42)	0.86 (0.51-1.46)	17	0.98 (0.50-1.93)	10	1.00 (0.39-2.57)
Q4: >188 (225)	17	0.49 (0.28-0.89)	0.49 (0.27-0.91)	11	0.62 (0.29-1.35)	5	0.45 (0.39-1.42)
P-trend		*0.02*	*0.02*		*0.21*		*0.18*
Men							
Q1: <66 (44)	30	1.0 (ref)	1.0 (ref)	22	1.0 (ref)	6	1.0 (ref)
Q2: 66-118 (89)	33	1.13 (0.69-1.86)	1.16 (0.70-1.92)	20	0.93 (0.50-1.74)	7	1.22 (0.40-3.70)
Q3: 119-198 (157)	24	0.78 (0.46-1.34)	0.84 (0.48-1.45)	18	0.87 (0.46-1.65)	1	0.16 (0.02-1.32)
Q4: >199 (241)	28	0.86 (0.51-1.44)	0.87 (0.51-1.48)	20	0.85 (0.45-1.60)	2	0.28 (0.05-1.46)
P-trend		*0.32*	*0.36*		*0.61*		*0.03*

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HR, hazard ratio; CI, confidence interval.

[a] Values are HR with 95% CI in quartiles (Q1-Q4) of EPA-DHA intake, using Q1 as the reference category.

[b] International Classification of Diseases (ICD-10) codes were I60-I66 and G45 for total stroke; I63, I65, I66, and G45 for ischemic stroke and I60-I62 for hemorrhagic stroke.

[c] Model 1: adjusted for age.

[d] Model 3: additionally adjusted for smoking, BMI, educational level, parental history of myocardial infarction, alcohol intake, total energy intake, dietary fiber, vitamin C, beta-carotene, saturated fatty acids, *trans*-fatty acids, monounsaturated fatty acids, linoleic acid, and alpha-linolenic acid.

The associations of EPA-DHA and fish consumption with ischemic stroke and total stroke were similar. The HR (95% CI) for ischemic stroke risk in the top quartile of EPA-DHA intake was 0.62 (0.29-1.35) for women and 0.85 (0.45-1.60) for men. However, confidence intervals were wider compared to total stroke and the associations were not significant in either men or women. Results for total and ischemic stroke with or without TIA were also similar (results not shown). Although we had a limited number of cases, the associations of EPA-DHA and fish with hemorrhagic stroke suggested also an inverse association.

Table 6.4 Associations of incident stroke by quartiles of fish consumption in 20,069 Dutch men and women[a]

	Total stroke[b]			Ischemic stroke		Hemorrhagic stroke	
Intake, g/d	Cases	Model 1[c]	Model 2[d]	Cases	Model 2[d]	Cases	Model 2[d]
Range (median)		HR (95% CI)	HR (95% CI)		HR 95% CI		HR 95% CI
Women							
Q1: <3.0 (1.0)	29	1.0 (ref)	1.0 (ref)	17	1.0 (ref)	6	1.0 (ref)
Q2: 3.0-7.2 (4.2)	34	1.21 (0.74-1.98)	1.25 (0.75-2.08)	20	1.25 (0.65-2.41)	12	1.97 (0.73-5.31)
Q3: 7.3-14.0 (9.8)	28	0.96 (0.57-1.61)	1.00 (0.59-1.71)	18	1.14 (0.58-2.24)	8	1.19 (0.41-3.52)
Q4: >14.0 (18.0)	15	0.48 (0.26-0.90)	0.49 (0.26-0.94)	9	0.54 (0.24-1.23)	5	0.67 (0.19-2.29)
P-trend		*0.01*	*0.01*		*0.09*		*0.20*
Men							
Q1: <3.3 (1.1)	32	1.0 (ref)	1.0 (ref)	22	1.0 (ref)	5	1.0 (ref)
Q2: 3.3-7.4 (4.3)	32	1.03 (0.63-1.68)	1.04 (0.63-1.72)	22	1.05 (0.57-1.93)	7	1.22 (0.40-3.70)
Q3: 7.5-14.0 (10.8)	24	0.68 (0.40-1.16)	0.73 (0.42-1.24)	17	0.77 (0.40-1.47)	3	0.16 (0.02-1.32)
Q4: >14.0 (17.6)	27	0.74 (0.44-1.23)	0.75 (0.44-1.26)	19	0.79 (0.42-1.48)	1	0.28 (0.05-1.46)
P-trend		*0.11*	*0.14*		*0.31*		*0.04*

Abbreviations: HR, hazard ratio; CI, confidence interval.

[a] Values are HR with 95% CI in quartiles (Q1-Q4) of fish intake, using Q1 as the reference category.

[b] International Classification of Diseases (ICD-10) codes were I60-I66 and G45 for total stroke; I63, I65, I66, and G45 for ischemic stroke and I60-I62 for hemorrhagic stroke.

[c] Model 1: adjusted for age.

[d] Model 2: additionally adjusted for smoking, BMI, educational level, parental history of myocardial infarction, alcohol intake, supplement use, total energy intake, dietary fiber, vitamin C, beta-carotene, saturated fatty acids, *trans*-fatty acids, monounsaturated fatty acids, linoleic acid, and alpha-linolenic acid.

Additional adjustment for systolic blood pressure did not change the associations. HRs (95% CI) for total stroke incidence in the top quartile of EPA-DHA were 0.50 (0.27-0.92) for women and 0.85 (0.50-1.46) for men. In the top quartile of fish consumption, HRs for total stroke were 0.74 (0.44-1.25) and 0.50 (0.26-0.95) for men and women, respectively. For the subgroup with information on physical activity (n=15,423), the full model with and without physical activity yielded similar results (results not shown).

Discussion

In this prospective cohort study from the Netherlands, a higher EPA-DHA and fish intake were associated with a lower stroke risk in women, with no differences between stroke types. For men, these associations were weaker and not statistically significant.

Misclassification of participants for fish or EPA-DHA intake may have occurred. However, correlations for EPA and DHA intake derived from the FFQ with levels in plasma cholesteryl esters were 0.32 and 0.41, which is comparable to other studies.[28] Furthermore, we excluded participants with a history of myocardial infarction or stroke and participants on cholesterol or

blood pressure lowering medication, because those participants may have changed their diets. We therefore consider potential misclassification at baseline random rather than dependent on disease outcome.

Nonfatal stroke events were assessed through probabilistic linkage with the national hospital discharge register. If we have missed events by this procedure, this is unlikely to be related to EPA-DHA or fish intake, and will therefore not have biased our results. In the Netherlands, brain imaging is used to identify stroke in 98% of the admitted stroke patients.[29] In contrast to most other studies on stroke, we also evaluated TIA (which comprised 42% of ischemic stroke cases), a less severe stroke event of which symptoms last <24h. Because the results for incident stroke with or without TIA were similar in our study, we included TIA to increase statistical power.

We observed a significant 51% lower risk of total stroke in women in the highest quartile of EPA-DHA (>188 mg/d), or fish (>14 g/d) intake. The difference with the bottom quartile corresponded to ~one portion of fish per week. Our associations for EPA-DHA were stronger than in the Nurses' Health Study. In that study, EPA-DHA intakes in quintile 3 (median 171 mg/d) and 4 (median 221 mg/d), which approximately represent our top quartile, were associated with a respectively 31% and 17% lower stroke risk, compared to the bottom quintile.[9] American cohort studies reported a 23% (HR: 0.77; 95% CI: 0.53-1.13)[8] and a 22% lower (HR: 0.78; 95% CI: 0.55-1.12)[9] stroke risk for women who consumed one fish meal per week compared to no fish[8] or less than once per month.[9] For women from the UK[12] and Sweden.[12,13] eating fish once or twice per week compared to less than once per week was associated with borderline significant lower stroke incidences of 26% (UK) and 13% (Sweden). In summary, cohort studies from Western countries have consistently shown that EPA-DHA and fish intake are inversely associated with stroke risk in women, with HRs varying between 0.5 and 0.8.

We found no significant association for EPA-DHA with stroke in men. In the Health Professionals Follow up Study, the largest male cohort, a 23% lower stroke risk (HR: 0.77; 95% CI: 0.52-1.14) was observed for an EPA-DHA intake of 200-400 mg/d vs. less than 50 mg/d after 12 years of follow-up.[10] In the Physicians' Health Study, however, EPA-DHA intake was not associated with 4-year incidence of stroke.[14] Although not significant, the HR of 0.75 for stroke in the top vs. bottom quartile of fish consumption in our male participants was in line with the borderline significant 26% lower stroke risk in the American health professionals consuming one fish meal per week compared to less than one per month.[10] Our stroke risk estimate of 0.75 was stronger than the male-specific estimate of 0.90 (95% CI: 0.78-1.04) for fish once per week vs. less than once per month from the meta-analysis of He *et al*.[19] That effect size estimate, however, was diluted by a large Chinese study that reported a positive association between fish intake and fatal stroke.[16] In men from the UK, eating fish (mainly processed and fried) once or twice per week compared to less than once per week was associated with a non-significant higher stroke risk.[12] To summarize, in cohort studies from Western countries with a similar range of intake compared to our study, inverse associations for men are less convincing than for women.

Concluding, evidence is accumulating that a higher EPA-DHA and fish intake is related to a lower stroke risk in women, while for men an inverse association could not be demonstrated. This gender difference cannot be explained by differences in stroke types as inverse associations were observed both for ischemic and hemorrhagic stroke. Furthermore, distributions of EPA-DHA and fish intake for men and women were similar. If this gender difference will be confirmed in other and larger studies, research is needed to clarify the physiologic difference of this epidemiologic finding.

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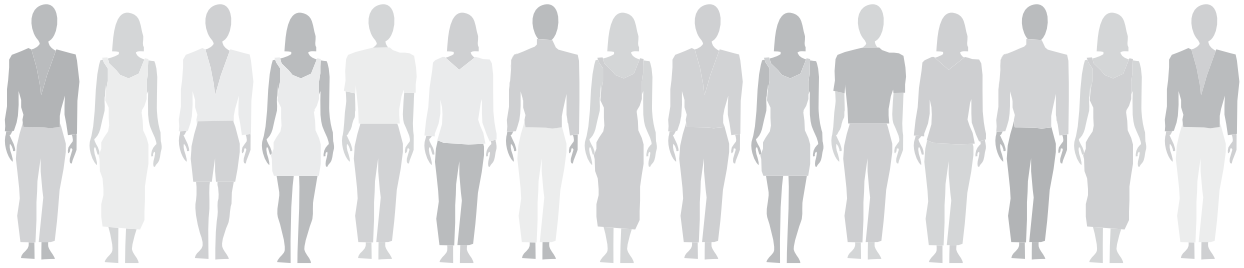

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N-6 and n-3 fatty acid cholesteryl esters in relation to fatal coronary heart disease in a Dutch adult population: a nested case-control study

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Abstract

Aim: Dietary polyunsaturated fatty acids (PUFA) are inversely related to coronary heart disease (CHD) in epidemiological studies. We examined the associations of plasma n-6 and n-3 PUFA in cholesteryl esters with fatal CHD in a nested case-control study.

Methods and results: We used data from two population-based cohort studies in Dutch adults aged 20-65 years. Blood sampling and data collection took place from 1987-1997 and subjects were followed for 8-19 years. We identified 279 incident cases of fatal CHD (235 fatal myocardial infarctions and 44 cardiac arrests) and randomly selected 279 controls, matched on age, gender, and enrollment date. Odds ratios (OR) with 95% confidence intervals (95% CI) were calculated per standard deviation (SD) increase of fatty acids in cholesteryl esters using multivariable conditional logistic regression models. After adjustment for confounders, the OR (95% CI) for fatal CHD per SD increase in plasma linoleic acid was 0.89 (0.74-1.06). Additional adjustment for plasma total cholesterol and systolic blood pressure attenuated this association (OR: 0.95; 95% CI: 0.78-1.15). Plasma arachidonic acid was not associated with fatal CHD (OR per SD: 1.11; 95% CI: 0.92-1.35). The ORs (95% CI) for fatal CHD for an SD increase in n-3 PUFA were 0.92 (0.74-1.15) for plasma alpha-linolenic acid and 1.06 (0.88-1.27) for plasma EPA-DHA.

Conclusion: In this Dutch adult population, arachidonic acid and n-3 PUFA in cholesteryl esters were not related to fatal CHD. Our data support findings from previous prospective studies showing a lower proportion of linoleic acid in plasma cholesteryl esters in CHD cases.

Introduction

Several reviews of prospective cohort studies and randomized trials suggest that the intake of n-6 and n-3 polyunsaturated fatty acids (PUFA) protect against coronary heart disease (CHD).[1-4] Linoleic acid, belonging to the n-6 PUFA family, is the most abundant PUFA in the diet and it is mainly obtained from vegetable oils, such as sunflower oil and soybean oil.[2] It is an essential fatty acid that can be elongated to arachidonic acid, which is also present in meat in small quantities.[5,6] Alpha-linolenic acid is an essential fatty acid of the n-3 PUFA family and is present in soybean, canola, and flaxseed oil.[2] Alpha-linolenic acid can be elongated to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Because these conversions takes place only to a limited extent (<8%),[7-9] EPA and DHA are mainly derived from the diet, through fish consumption.[2]

Biomarkers of dietary intake are widely used in epidemiological studies.[10,11] They are considered to provide a more accurate measure of intake than dietary records or questionnaire data, especially when the nutrient of interest varies widely within foods and food groups and when food composition tables are inaccurate for that specific nutrient.[12] Furthermore, biomarkers are not dependent on a person's ability to recall dietary intakes. Fatty acids can be measured as free fatty acids in serum (or plasma), as components of triglycerides, phospholipids, cholesteryl esters, erythrocyte membranes, platelets, or in adipose tissue from various sites.[13] Cholesteryl esters are found in plasma lipoproteins and reflect dietary intake of PUFA during the previous weeks.[14,15]

Harris *et al*.[16] performed a meta-analysis of 25 (nested) case-control studies and prospective cohort studies on tissue fatty acid composition and risk of CHD published until 2006. Harris *et al*. showed that long-chain n-3 PUFA tissue concentrations, especially DHA, were inversely associated with fatal CHD. However, in their meta-analysis crude data of PUFA levels were pooled, i.e. potential confounders were not taken into account. Furthermore, adipose tissue and various plasma and serum fractions were combined.

We investigated the associations of n-6 and n-3 PUFA, measured in plasma cholesteryl esters with the risk of fatal CHD in a prospective case-control study of Dutch adults, adjusted for confounders. Additionally, we performed a meta-analysis of nested case-control and cohort studies on plasma PUFA measured in cholesteryl esters in relation to CHD.

Methods

Design and study populations

We conducted a nested case-control study using two similar consecutive Dutch population-based cohorts. The nested case-control design is considered an efficient alternative to a full-cohort analysis.[17] Baseline blood samples and information on lifestyle, and cardiovascular risk

factors were collected in 35,475 subjects aged 20-59 years during 1987-1991 in the Monitoring Project on Cardiovascular Disease Risk Factors (subsequently referred to as MP-1)[18,19] and in 20,641 subjects aged 20-65 years during 1993-1997 in the Monitoring Project on Risk Factors for Chronic Diseases (MP-2).[20] The surveys comply with the Declaration of Helsinki and the protocols were approved by the Academic Hospital Leiden and the Medical Ethics Committees of TNO Prevention and Health, Leiden, The Netherlands. Written informed consent was obtained from each participant. For 7,754 participants who participated in both cohorts, we used the more recent MP-2 data. In addition, we excluded participants with a history of myocardial infarction (MI) or stroke at baseline, resulting in 26,987 participants in MP-1 and 21,335 participants in MP-2.

Vital status was checked through linkage with the national population register. Participants were followed for cause-specific mortality through linkage with Statistics Netherlands. Fatal CHD included fatal myocardial infarction (MI; I21, I22) and fatal cardiac arrest (CA; I46), according to the International Classification of Diseases (ICD-10, WHO). For causes of death coded until January 1, 1996, corresponding ICD-9 codes were used. Participants were followed until fatal CHD, death, date of loss-to-follow-up (predominantly because of emigration) or 1 January 2006, whichever came first.

All cases of fatal MI and fatal CA that occurred during follow-up (median 12.5 years, range 8-19 years) were identified. For each case (n=232 in MP-1 and 69 in MP-2), one control from the same cohort was selected based on incidence density sampling to reduce the likelihood of biased results.[21,22] Controls were selected from those persons under study who survived at least as long as the index case. A person was eligible to serve as a control for multiple cases at a given moment in time and could serve both as control and case. Cases were individually matched to controls on age (± 0.5y), gender, and date of entry in the cohort (± 0.5y). Plasma was available for 222 case-control pairs of MP-1 and 57 pairs of MP-2. In MP-1, five participants were selected as a control twice and four participants served both as a control and as a case. In MP-2, 1 participant was selected both as case and control.

Measurement of n-3 PUFA in plasma cholesteryl esters

Participants donated nonfasting blood at baseline. EDTA-plasma of MP-1 was stored at -30°C and EDTA-plasma of MP-2 was stored at -80°C until analyzed. Fatty acids were measured in plasma cholesteryl esters by gas chromatography, as described previously.[23] In short, to isolate cholesteryl esters, lipids from EDTA plasma were dissolved and separated by solid phase extraction silica columns (Chrompack, Middelburg, The Netherlands). The fatty acids were identified by comparison with known standards (Nu-chek prep, Inc. Elysian, MN, USA). Fatty acids were expressed as mass percentages of total fatty acid methyl esters (g/100g). A quality control plasma pool was analyzed in duplicate in each run. Coefficients of variation of the controls (intra and inter assay combined) ranged between 3 and 3.5%. Laboratory

technicians were blinded to the status of the samples. Cases and controls were randomly distributed over the runs.

Data collection on risk factors

The baseline measurements were previously described in detail by Verschuren *et al*.[18,20] Body weight, height, and blood pressure were measured by trained research nurses. Hypertension was defined as a systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or the use of blood pressure lowering medication. Nonfasting plasma was analyzed for total and high-density lipoprotein (HDL) cholesterol, and hypercholesterolemia was defined as plasma total cholesterol ≥6.5 mmol/l or use of cholesterol lowering medication. Self-administered questionnaires were used to assess the prevalence of diabetes, history of MI or stroke, medication use, parental history of MI, educational level, and cigarette smoking. Alcohol intake was calculated in glasses/d and was categorized as no intake, low to moderate intake (men ≤2 and women ≤1 glasses/d), or high intake (men >2 and women >1 glasses/d).

Statistical analysis

In descriptive analyses, we compared the prevalence of risk factors and mean levels (± SD) of plasma fatty acids between cases and controls, stratified for cohort. The significance of differences in crude means or frequencies of risk factors were assessed by paired t-test for continuous variables and by the Wilcoxon signed-rank test for categorical variables. We used conditional logistic regression models to calculate odds ratios (OR) with 95% confidence intervals (95% CI) for the association of plasma levels of linoleic acid, arachidonic acid, alpha-linolenic acid, EPA, DHA, and EPA-DHA with fatal CHD. The analyses were repeated within the two separate cohorts. ORs and 95% CI for fatal CHD were calculated per SD increase in the plasma fatty acids, based on the distribution of controls.

In model 1, we adjusted for the matching factors age, gender, cohort, and enrollment date. In model 2, we additionally adjusted for current cigarette smoking (yes/no), body mass index (kg/m^2), alcohol intake (no, low to moderate, or high), high educational level (completed higher vocational training or university). In model 3 we also adjusted for systolic blood pressure (mmHg) and plasma total cholesterol (mmol/l). Two-sided *P*-values <0.05 were considered to be statistically significant. Descriptive statistics and logistic regression analyses were performed with Statistical Analysis Software (SAS), version 9.2.

We performed a dose response meta-analysis of prospective studies (cohort studies or nested case-control studies) that measured cholesteryl ester PUFA status in relation to CHD risk including the estimates of the two separate cohorts of the current study. We identified five publications with relative risks on cholesteryl ester PUFA in relation to fatal and nonfatal CHD,[24-28] of which one could not be used due to missing data on fatty acid levels.[26] In the

original publication of Warensjö *et al*.[28] the endpoint was fatal cardiovascular disease. Data on CHD were kindly provided by the authors upon request. We used STATA version 11.0 (STAT Corp, College Station, TX) for meta-analyses using the METAN command. The generalized least-squares method for trend estimation of summarized dose-response data was used to calculate a relative risk for a certain unit of the exposure based on the Greenland and Longnecker method.[29] Each study was weighted by the inverse of its variance, including both the within and between study variance. Between-study heterogeneity was assessed via the I2 statistic, which expresses the percentage of variation attributable to between-study heterogeneity.[30] Random effects pooling were conducted according to DerSimonian and Laird.[31] We visualized and summarized the associations between different PUFA and CHD outcomes in forest plots.

Table 7.1 Baseline characteristics of 279 fatal coronary heart disease cases and 279 matched controls controls[a,b]

	MP-1			MP-2		
	Cases (n=222)	Controls (n=222)	*P*-value[c]	Cases (n=57)	Controls (n=57)	*P*-value[c]
Male gender, %	70	70	–	79	79	–
Age, y	50.5 ± 7.4	50.5 ± 7.5	–	51.7 ± 7.1	51.8 ± 7.2	–
Body mass index, kg/m^2	26.9 ± 4.7	26.0 ± 3.9	0.02	27.7 ± 4.8	26.2 ± 3.8	0.07
Smoking, %						
Never	18	32	–	18	35	–
Former	21	30	–	26	39	–
Current	61	38	<0.0001	56	26	0.002
Alcohol consumption, %						
No intake	38	34	–	23	12	–
Low to moderate	34	36	–	44	67	–
High	27	30	0.29	33	21	0.90
High educational level,[d] %	7	14	0.008	21	18	0.82
Parental history of myocardial infarction, %	9.5	8.7	0.87	5.3	5.3	1.00
Diabetes mellitus, %	5.0	2.7	0.33	5.3	0	0.25
Systolic blood pressure, mm Hg	134.5 ± 21.0	125.5 ± 15.6	<0.0001	138.8 ± 20.3	126.8 ± 17.1	0.001
Diastolic blood pressure, mm Hg	83.4 ± 12.4	78.6 ± 9.7	<0.0001	85.6 ± 12.1	79.7 ± 9.9	0.002
Blood pressure lowering medication, %	14.9	9.0	0.06	19.3	10.5	0.23
Hypertension, %	46	28	<0.0001	56	33	0.002
Plasma total cholesterol,[e] mmol/l	6.5 ± 1.3	5.9 ± 1.1	<0.0001	5.8 ± 1.0	5.7 ± 1.0	0.83
Plasma HDL-cholesterol,[e] mmol/l	1.1 ± 0.3	1.2 ± 0.3	0.002	1.2 ± 0.4	1.3 ± 0.3	0.15
Cholesterol lowering medication, %	1.8	0.5	0.25	1.8	0	1.00
Hypercholesterolemia, %	42	27	0.001	26	19	0.42

[a] Values are means ± SD, unless indicated otherwise.
[b] Controls were matched on age, gender, cohort, and enrollment date.
[c] Paired t-test for linear values and Wilcoxon signed-rank test for proportions.
[d] Completed higher vocational training or university.
[e] Nonfasting.

Results

Nested cases-control study of plasma cholesteryl fatty acids and fatal CHD

Cases comprised 235 fatal MI (187 from MP-1 and 48 from MP-2) and 44 cardiac arrest events (35 from MP-1 and 9 from MP-2). Cases and matched controls from MP-2 were on average around 51 years old and 79% was male. Compared to MP-2, cases and controls from MP-1 had a similar age, but consisted of fewer males (70%). In both cohorts, cases had a higher body mass index, smoked more, more often used anti-hypertensive medication, and had higher blood pressure levels than controls. Cases of MP-1 also had higher plasma total cholesterol levels compared to controls (**Table 7.1**).

Table 7.2 shows fatty acid levels for CHD cases and matched controls. The levels of linoleic acid, arachidonic acid, alpha-linolenic acid, and EPA-DHA were all lower in MP-1 as compared to MP-2. Linoleic acid values were (non-significantly) lower in cases compared to controls, although this was not statistically significant. The other fatty acid levels did not differ between cases and controls.

In the crude model and after adjusting for smoking, body mass index, educational level, and alcohol intake (model 2), linoleic acid status was borderline significantly inversely associated with fatal CHD. In model 2, the ORs (95% CI) for fatal CHD per SD increase in linoleic acid was

Table 7.2 Fatty acid proportions in plasma cholesteryl esters in 279 Dutch fatal CHD cases and 279 matched controls[a,b]

Fatty acids (g/100g)[c]		Cases	Controls	*P*-value[d]
MP-1		**n=222**	**n=222**	
Linoleic acid	C18:2n-6	42.9 ± 7.0	43.8 ± 6.3	0.15
Arachidonic acid	C20:4n-6	3.8 ± 1.1	3.9 ± 1.2	0.54
Alpha-linolenic acid	C18:3n-3	0.39 ± 0.13	0.38 ± 0.14	0.56
EPA	C20:5n-3	0.59 ± 0.45	0.59 ± 0.42	0.73
DHA	C22:6n-3	0.33 ± 0.16	0.33 ± 0.15	0.59
EPA-DHA	C20:5n-3+C22:6n-3	0.92 ± 0.57	0.91 ± 0.54	0.996
MP-2		**n=57**	**n=57**	
Linoleic acid	C18:2n-6	52.9 ± 5.3	54.4 ± 4.8	0.14
Arachidonic acid	C20:4n-6	6.8 ± 1.6	6.4 ± 1.6	0.18
Alpha-linolenic acid	C18:3n-3	0.51 ± 0.15	0.51 ± 0.14	0.95
EPA	C20:5n-3	0.93 ± 0.64	0.89 ± 0.57	0.88
DHA	C22:6n-3	0.54 ± 0.22	0.52 ± 0.22	0.46
EPA-DHA	C20:5n-3+C22:6n-3	1.48 ± 0.82	1.41 ± 0.74	0.69

[a] Fatty acid levels are expressed as mass percentages (g/100g).

[b] Controls were matched on age, gender, cohort, and enrollment date.

[c] Fatty acid levels are expressed as means ± SD.

[d] Paired t-test, log transformed values were used for EPA, DHA, and EPA-DHA.

0.89 (0.74-1.06). Additional adjustment for plasma total cholesterol and systolic blood pressure (model 3) attenuated the estimate. Plasma arachidonic acid was not associated with fatal CHD. The ORs (95% CI) for fatal CHD for an SD increase in n-3 PUFA were 0.92 (0.74-1.15) for plasma alpha-linolenic acid and 1.06 (0.88-1.27) for plasma EPA-DHA (**Table 7.3**).

Meta-analysis of prospective studies on cholesteryl ester PUFA in relation to CHD

For the meta-analysis, we pooled the current data with results of two nested case-control studies from the USA[22, 23] and two cohort studies from Finland and Sweden on cholesteryl ester PUFA in relation to fatal and nonfatal CHD.[25,26] The mean baseline age ranged from 50-60 years and the mean follow-up time ranged from 5-34 years between studies. Three studies comprised only

Table 7.3 Associations between plasma cholesteryl ester fatty acids and fatal CHD, matched by age, gender, cohort, and enrollment date[a]

	Model 1[b]	Model 2[c]	Model 3[d]
	OR (95% CI)	OR (95% CI)	OR (95% CI)
Combined cohorts	**n=558**		
Linoleic acid	0.86 (0.74-1.00)	0.89 (0.74-1.06)	0.95 (0.78-1.15)
Arachidonic acid	1.00 (0.85-1.18)	1.06 (0.88-1.27)	1.11 (0.92-1.35)
Alpha-linolenic acid	1.05 (0.87-1.25)	0.97 (0.79-1.19)	0.92 (0.74-1.15)
EPA	1.02 (0.88-1.20)	1.07 (0.90-1.26)	1.04 (0.86-1.24)
DHA	1.02 (0.87-1.20)	1.09 (0.91-1.31)	1.12 (0.92-1.36)
EPA-DHA	1.03 (0.88-1.20)	1.08 (0.91-1.27)	1.06 (0.88-1.27)
MP-1	**n=444**		
Linoleic acid	0.88 (0.75-1.05)	0.90 (0.74-1.10)	0.97 (0.78-1.21)
Arachidonic acid	0.95 (0.79-1.13)	1.02 (0.83-1.25)	1.08 (0.87-1.34)
Alpha-linolenic acid	1.06 (0.87-1.31)	1.01 (0.80-1.26)	0.93 (0.72-1.19)
EPA	1.01 (0.85-1.21)	1.06 (0.87-1.28)	1.02 (0.83-1.25)
DHA	1.00 (0.84-1.20)	1.07 (0.88-1.30)	1.11 (0.89-1.37)
EPA-DHA	1.01 (0.85-1.20)	1.06 (0.88-1.29)	1.04 (0.85-1.28)
MP-2	**n=114**		
Linoleic acid	0.77 (0.54-1.10)	0.80 (0.53-1.23)	0.83 (0.53-1.32)
Arachidonic acid	1.32 (0.88-1.96)	1.26 (0.79-2.03)	1.29 (0.78-2.12)
Alpha-linolenic acid	0.99 (0.68-1.45)	0.87 (0.56-1.37)	1.01 (0.61-1.70)
EPA	1.08 (0.76-1.51)	1.11 (0.72-1.70)	1.06 (0.65-1.74)
DHA	1.15 (0.77-1.71)	1.22 (0.72-2.05)	1.15 (0.65-2.05)
EPA-DHA	1.09 (0.77-1.55)	1.13 (0.73-1.77)	1.08 (0.65-1.80)

Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

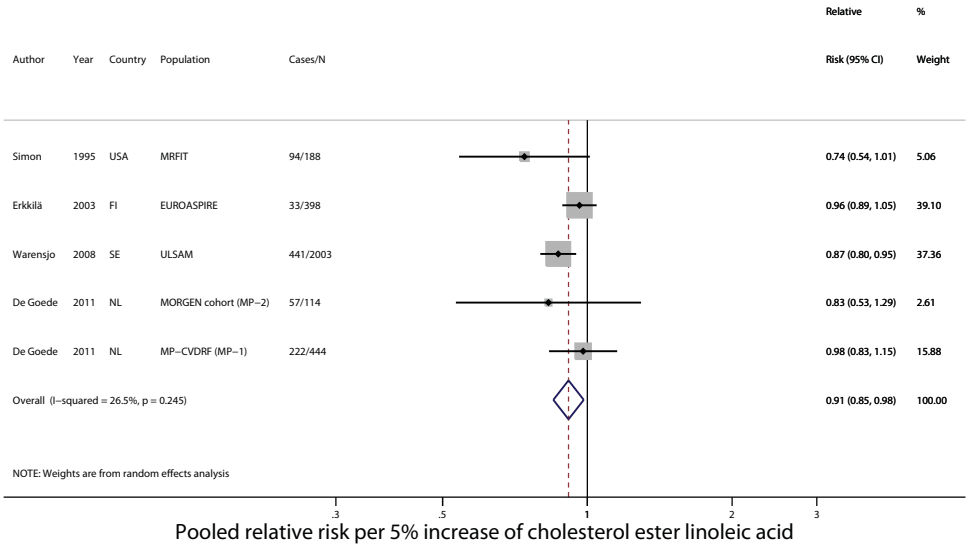
[a] Values are odds ratios (95% CI) per standard deviation increase, based on conditional logistic models.

[b] Crude model, matched for age, gender, cohort, and enrollment date.

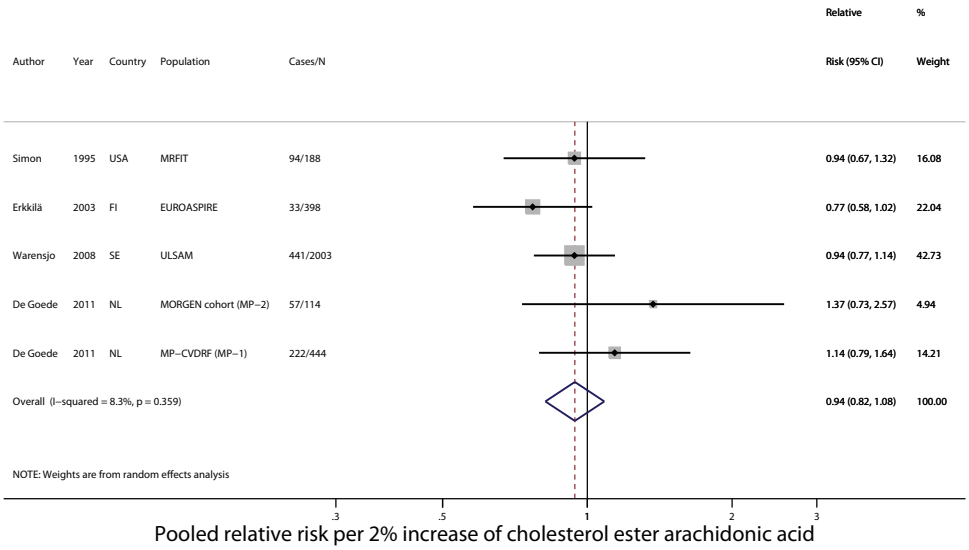
[c] Model 1 with additional adjustment for smoking, BMI, education level, alcohol intake.

[d] Model 2 with additional adjustment for systolic blood pressure, total cholesterol.

men[24,25] and the other included men and women. For MP-1 and MP-2 we used the estimates of model 3, as that model was most comparable to the data of the other included studies. After pooling all studies, a 5% higher linoleic acid level was associated with a 9% lower risk (relative risk: 0.91; 95% CI: 0.84-0.98) of CHD. The other fatty acids were not associated with CHD. For DHA status we observed significant heterogeneity (P<0.001) (**Figure 7.1**). Exclusion of the study of Erkkilä *et al*., which was the only study with coronary patients, resulted in a pooled OR (95% CI) of 1.09 (0.95-1.11) without heterogeneity (P=0.49).

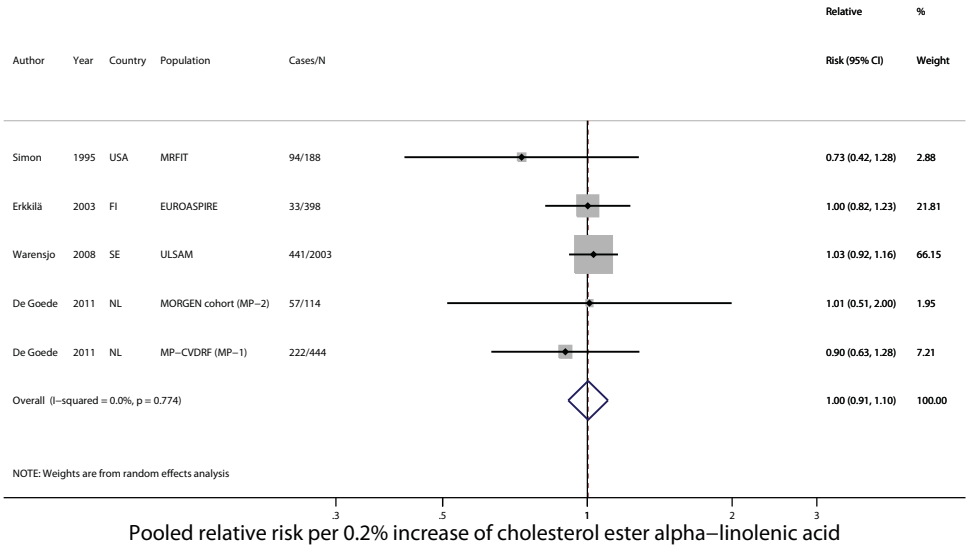


Pooled relative risk per 5% increase of cholesterol ester linoleic acid

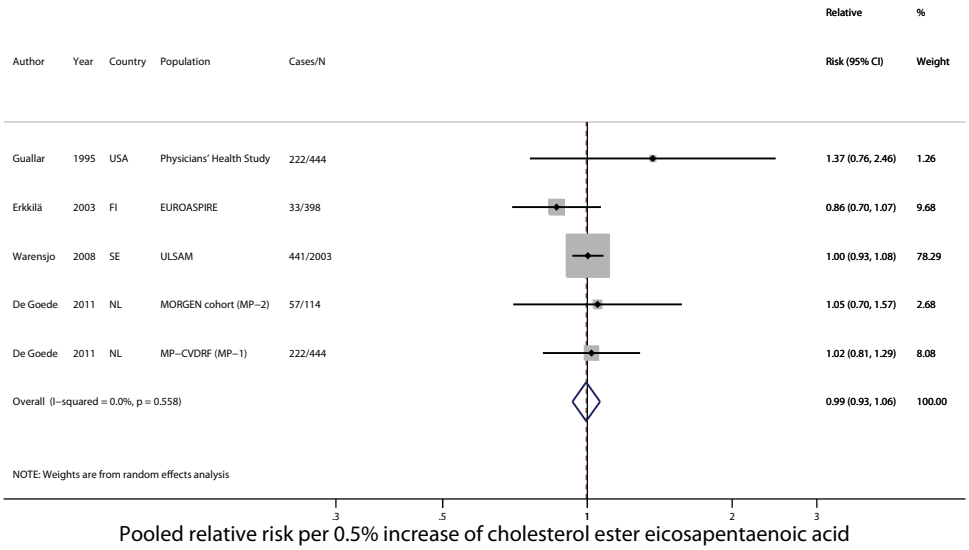


Pooled relative risk per 2% increase of cholesterol ester arachidonic acid

Figure 7.1a-b Pooled relative risk of cholesterol ester n-6 and n-3 PUFA.



Pooled relative risk per 0.2% increase of cholesterol ester alpha−linolenic acid



Pooled relative risk per 0.5% increase of cholesterol ester eicosapentaenoic acid

Figure 7.1c-d Pooled relative risk of cholesterol ester n-6 and n-3 PUFA.

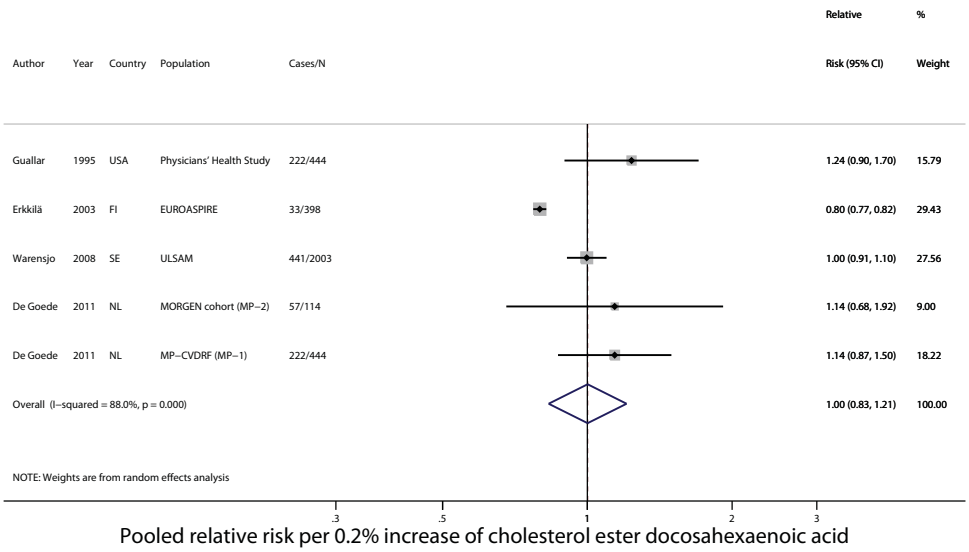


Figure 7.1e Pooled relative risk of cholesterol ester n-6 and n-3 PUFA.

Discussion

In a nested case-control study in Dutch adults we observed an inverse, but statistically non-significant association between plasma cholesteryl ester linoleic acid levels and fatal CHD. When we pooled these data with those from similar prospective studies in a meta-analysis, a 5% higher linoleic acid level was related to a significant 9% lower CHD risk. Arachidonic acid and the n-3 PUFA alpha-linolenic acid, EPA, and DHA were not associated with CHD risk in the present study and in the meta-analysis.

A limitation of our study could be that the blood samples were stored for 18-23 years for MP-1 and 12-17 years for MP-2, which may have affected the quality of plasma fatty acids. However, storage up to 10 years at -80°C did not significantly influence serum cholesteryl ester fatty acid profiles in a recent validation study.[32] Although the n-6 and n-3 PUFA levels of the (older) MP-1 samples were considerably lower than those of the MP-2 samples, we do not expect that the values were differentially lower for cases compared to controls. The number of detected fatty acids (15-20) and the percentage of unknown fractions (rule of thumb <5%) were as expected for both cohorts. Furthermore, potential measurement error will have been random because the plasma samples of cases and controls were identically handled and analyzed in random order, and lab technicians were blinded for disease outcome. A strength of the present analysis was that we used two similar, large population-based cohort studies, with almost complete mortality follow-up.

The present nested case-control study showed an inverse, but statistically non-significant association between plasma cholesteryl ester linoleic acid levels and fatal CHD. However, a 5% higher linoleic acid level was related to a significant 9% lower CHD risk (OR: 0.91; 95% CI: 0.84-0.98) in a meta-analysis in which we combined our findings with data from similar prospective studies. In the meta-analysis of Harris *et al*.,[16] linoleic acid was not associated with CHD risk, based on a pooled estimate of seven prospective studies with various blood fractions. Plasma arachidonic acid did not predict CHD in our nested case-control study and meta-analysis, which was in agreement with Harris *et al*.[16] In our nested case-control study and meta-analysis, we observed no association of cholesteryl ester alpha-linolenic acid or EPA-DHA with CHD, whereas Harris *et al*.[16] observed a borderline significantly lower alpha-linolenic acid status in CHD cases. Furthermore, DHA, but not EPA, was significantly inversely associated with CHD in the subgroup of prospective studies in the meta-analysis of Harris *et al*.[16]

The current meta-analysis and the one of Harris *et al*.[16] showed different results, mainly for linoleic acid and DHA. Some differences in design could be responsible for this. Although Harris *et al*. combined data of a large number of studies, 16 out of the 25 studies had a classical case-control design (based on prevalent cases), which is more prone to reverse-causation and selection bias. Seven studies (case-control studies only) were based on adipose tissue samples. The other 18 used various blood fractions, such as phospholipids, cholesteryl esters, and erythrocytes, which could cause substantial heterogeneity in meta-analysis results. Finally, the analysis was based on crude PUFA levels. Potential confounding e.g. by body mass index and smoking, which appeared to be strong confounders in the present analysis, may partly explain discrepant results between the two meta-analyses.

Linoleic acid is by far the most important fatty acid in cholesteryl esters, followed by oleic acid, palmitic acid, and arachidonic acid.[33] In contrast, the concentrations of n-3 PUFA are very low. Therefore, in the n-3 PUFA, the variation between persons was probably small compared to the within-person variation. An American validation study reported that short and long-term reliability coefficients i.e. the ratio of between-person variance to total variance were >0.7 for cholesteryl ester linoleic acid, whereas these coefficients ranged between 0.4-0.5 for fatty acids that composed <1% of total cholesteryl ester fatty acids. The variance of the method was only <5% of the total variance.[34] A low between to within-person variation ratio will hamper finding significant associations between these fatty acids and fatal CHD. This is probably the reason why the associations between these PUFA and fatal CHD are inconsistent (**Figure 7.1**).

In conclusion, our data support the previously reported inverse association between linoleic acid in plasma cholesteryl esters and CHD risk. For plasma cholesteryl ester levels of n-3 PUFA, however, no relations with CHD risk were found in our prospective study and meta-analysis, which raises concern regarding the validity of these biomarkers of intake for epidemiological studies.

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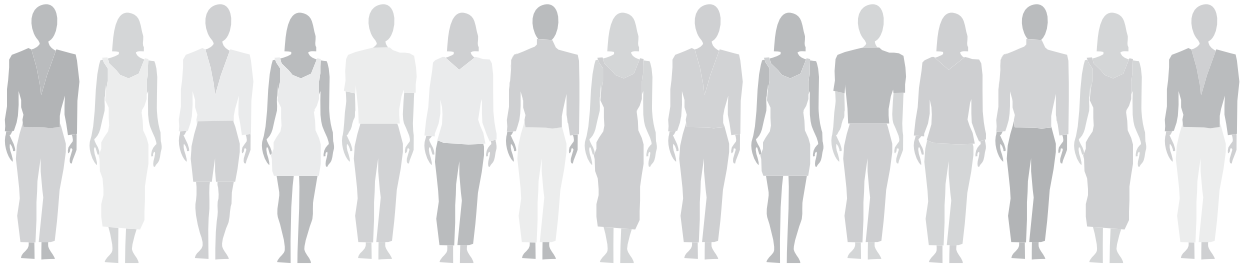

8

N-6 and n-3 fatty acid cholesteryl esters in relation to incident stroke in a Dutch adult population: a nested case-control study

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Abstract

Background and aims: There are few prospective studies on fatty acid status in relation to incident stroke, with inconsistent results. We assessed the associations of plasma n-6 and n-3 PUFA in cholesteryl esters with the risk of total stroke and stroke subtypes in Dutch adults.

Methods and results: We conducted a nested case-control study using data from a population-based cohort study in adults aged 20-65 years. Blood sampling and data collection took place during 1993-1997 and subjects were followed for 8-13 years. We identified 179 incident cases of stroke and 179 randomly selected controls, matched on age, gender, and enrollment date. Odds ratios (OR) with 95% confidence intervals (95% CI) were calculated per standard deviation (SD) increase of PUFA in cholesteryl esters using multivariable conditional logistic regression. Cases comprised 93 ischemic, 50 hemorrhagic, and 36 unspecified strokes. The n-6 PUFA linoleic acid and arachidonic acid contributed ~55% and ~6.5% respectively to total plasma fatty acids, whereas the n-3 PUFA alpha-linolenic acid contributed ~0.5% and eicosapentaenoic acid plus docosahexaenoic acid (EPA-DHA) ~1.3%. After adjustment for confounders, n-6 and n-3 PUFA were not associated with incident total stroke or stroke subtypes. The OR (95% CI) for total stroke was 0.95 (0.74-1.23) per SD increase in linoleic acid and 1.02 (0.80-1.30) per SD increase in arachidonic acid. ORs (95% CI) for total stroke were 0.94 (0.72-1.21) for alpha-linolenic acid and 1.16 (0.94-1.45) for EPA-DHA.

Conclusion: In the present study, plasma n-6 or n-3 fatty acids were not related to incident stroke or stroke subtypes.

Introduction

Worldwide, stroke is the second largest cause of death and a major cause of long-term disability.[1,2] Stroke was the third cause of burden of disease expressed in disability adjusted life years (DALY) in middle and high income countries in 2004, leading to substantial health care costs.[1] A healthy lifestyle and diet are of utmost importance for the primary prevention of cardiovascular diseases, including stroke.[3-5] Polyunsaturated fatty acids (PUFA) may influence the risk of stroke, but data on biomarkers of PUFA intake in relation to stroke risk are lacking.

Linoleic acid, belonging to the n-6 PUFA family, is the most abundant PUFA in the diet and it is mainly obtained from vegetable oils, such as sunflower oil and soybean oil.[6] It is an essential fatty acid that can be elongated to arachidonic acid, which is also present in meat in small quantities.[7,8] Alpha-linolenic acid is an essential fatty acid of the n-3 PUFA family and is present in soybean, canola, and flaxseed oil.[6] Alpha-linolenic acid can be elongated to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Because these conversions only take place to a limited extent,[9] EPA and DHA are mainly derived from the diet, through fish consumption.[6]

Biomarkers of dietary intake are widely used in epidemiological studies.[10,11] They are considered to be a more accurate measure of intake than dietary records or questionnaire data, especially when the nutrient of interest varies widely within foods and food groups and when food composition tables are inaccurate for that specific nutrient.[12] Furthermore, biomarkers are not dependent on a person's ability to recall dietary intakes. Fatty acids can be measured as free fatty acids in serum (or plasma), as components of circulating triglycerides, erythrocyte membranes, platelets, phospholipids or cholesteryl esters, or in adipose tissue from various sites.[13] Cholesteryl esters are found in plasma lipoproteins and reflect dietary intake of PUFA during the previous weeks.[14,15] Whole serum, serum fractions, and erythrocytes also reflect a relatively short-term intake (between days and months). In long-term observational studies, adipose tissue is considered the best choice to assess habitual fatty acid intake, because it reflects the intake of fatty acids during the previous months to years.[10,15] However, blood tissue is most widely used in observational studies because of its accessibility and the assumption that individuals do not make drastic short-term diet changes.[10]

There are only a few prospective studies on fatty acid status in relation to incident stroke.[16-18] In a Japanese,[17] but not in an American[16] nested case-control study, total serum linoleic acid and arachidonic acid[16] were inversely associated with incident stroke. Alpha-linolenic acid in serum cholesteryl esters and phospholipids was inversely associated with stroke risk in the American,[16] but not in the Japanese study.[17] In Japan, however, both fatty acid intake and stroke incidence are very different compared to Western countries.[17] A Swedish nested case-control study found a borderline positive association of EPA-DHA in erythrocytes with ischemic stroke in men but not in women, whereas EPA-DHA status was not associated with total stroke in men or in women.[18] In the Japanese and American studies, EPA and DHA were not related to stroke risk.

We investigated the associations of n-6 and n-3 fatty acids, measured in plasma cholesteryl esters with the risk of total stroke and stroke subtypes in a nested case-control study of Dutch adults.

Methods

Study population

We conducted a nested case-control study in the Monitoring Project on Risk Factors for Chronic Diseases (MORGEN study), a Dutch population-based cohort study. Blood samples and information on lifestyle, and cardiovascular risk factors were collected at baseline (1993-1997) in 21,953 subjects aged 20-65 years.[19,20] The survey complied with the Declaration of Helsinki and the protocol was approved by the Medical Ethics Committees of TNO Prevention and Health, Leiden. Written informed consent was obtained from each participant. We excluded participants without dietary information, participants with a history of myocardial infarction or stroke at baseline, resulting in 21,335 participants.

Vital status was checked through linkage with the national population register. Participants were followed for cause-specific mortality, including fatal stroke, through linkage with Statistics Netherlands. Information on nonfatal stroke was obtained from the national hospital discharge register as described in more detail elsewhere.[21] It has been shown that on the national level data from the Dutch hospital discharge register can be uniquely matched to a person for at least 88% of the hospital admissions.[21] Total stroke included I60-66, ischemic stroke included I63, I65, and I66, and hemorrhagic stroke included I60-I62 according to the International Classification of Diseases (ICD-10, WHO). For hospital admissions and for causes of death coded until January 1, 1996, corresponding ICD-9 codes were used. Participants were followed until incident stroke, death, date of loss-to-follow-up (predominantly because of emigration) or 1 January 2006, whichever came first.

All cases of incident stroke (n=200) that occurred during 8-13 years (median: 10.5 years) of follow-up were identified. For each case, one control was selected based on incidence density sampling.[22,23] Controls were selected from those persons under study who survived at least as long as the index case. A person was eligible to serve as a control for multiple cases at a given moment in time and could serve both as control and case. Cases were individually matched on age (± 0.5y), gender, and enrollment date (± 0.5y). Plasma was available for 179 case-control pairs. Five participants were selected as a control twice. One participant served both as a control and as a case.

Measurement of plasma n-3 PUFA in plasma cholesteryl esters

Participants donated nonfasting blood at baseline. EDTA-plasma was stored at -80°C until analyzed in 2010. Fatty acids were measured in plasma cholesteryl esters by gas chromatography, as described previously.[24] In short, to isolate cholesteryl esters, lipids from EDTA plasma were dissolved and separated by solid phase extraction silica columns (Chrompack, Middelburg, The Netherlands). The fatty acids were identified by comparison with known standards (Nu-chek prep, Inc. Elysian, MN, USA). Fatty acids were expressed as mass percentages of total fatty acid methyl esters (g/100g). A quality control plasma pool was analysed in duplicate in each run. Coefficients of variation of the controls (intra and inter assay combined) ranged between 3 and 3.5%. Laboratory technicians were blinded to the status of the samples. Cases and controls were randomly distributed over the runs.

Data collection on risk factors

The baseline measurements were previously described in detail by Verschuren *et al*.[25] Body weight, height, and blood pressure were measured at baseline by trained research nurses. Hypertension was defined as a systolic blood pressure ≥140 mmHg, a diastolic blood pressure ≥90 mmHg, or the use of blood pressure lowering medication. Nonfasting plasma was analyzed for total and high-density lipoprotein (HDL) cholesterol, and hypercholesterolemia was defined as plasma total cholesterol ≥6.5 mmol/l or the use of cholesterol lowering medication. Self-administered questionnaires were used to assess the prevalence of diabetes, history of myocardial infarction or stroke, medication use, parental history of myocardial infarction, educational level, and cigarette smoking. Alcohol intake (based on a food frequency questionnaire[26,27]) was calculated in glasses/d and was categorized as no intake, low to moderate intake (men ≤2 and women ≤1 glasses/d), or high intake (men >2 and women >1 glasses/d).

Statistical analysis

In descriptive analyses, we compared the prevalence of risk factors and mean levels (±SD) of plasma fatty acids between cases and controls. The significance of differences in crude means or frequencies of risk factors were assessed by paired t-test for continuous variables and Wilcoxon signed-rank test for categorical variables. Correlations between the different types of fatty acids in plasma were assessed with the Spearman rank correlation test.

We used conditional logistic regression models to calculate odds ratios (OR) with 95% confidence intervals (95% CI) for the association of plasma levels of linoleic acid, arachidonic acid, alpha-linolenic acid, and EPA-DHA with incidence of stroke. The analyses were repeated for stroke subtypes, i.e. ischemic stroke and hemorrhagic stroke. ORs and 95% CI for stroke

were calculated per SD increase in the plasma fatty acids, based on the distribution of controls. In model 1, we adjusted for the matching factors age, gender, and enrollment date. In model 2, we additionally adjusted for current cigarette smoking (yes/no), body mass index (kg/m^2), alcohol intake (none, low to moderate or high), high educational level (completed higher vocational training or university), parental history of myocardial infarction (yes/no; for father <55 y and for mother <65 y), presence of diabetes mellitus (yes/no), hypertension (yes/no), hypercholesterolemia (yes/no). Two-sided *P*-values ≤0.05 were considered statistically significant. All statistical analyses were performed with Statistical Analysis Software (SAS), version 9.2.

Results

Cases comprised 93 ischemic strokes, 50 hemorrhagic strokes, and 36 unspecified strokes. Due to matching, case and control participants had a similar mean age of around 50 years and 53% of both cases and controls were male. Cases smoked more, were lower educated, had higher blood pressures, and had more often hypercholesterolemia or diabetes mellitus (**Table 8.1**).

Table 8.1 Characteristics of 179 Dutch stroke cases and 179 matched controls[a,b]

	Cases (n=179)	Controls (n=179)	*P-value*[c]
Male gender, %	53	53	-
Age, y	50.1 ± 9.5	50.0 ± 9.5	-
Body mass index, kg/m^2	25.8 ± 4.1	25.9 ± 4.3	*0.74*
Smoking, %			
Never	21	40	-
Former	30	35	-
Current	49	26	*<0.0001*
Alcohol consumption, %			
Low	20	11	-
Moderate	49	63	-
High	31	26	*0.51*
High educational level,[d] %	12	22	*0.01*
Diabetes mellitus, %	5.6	0.6	*0.001*
Systolic blood pressure, mm Hg	132.1 ± 20.2	126.1 ± 16.1	*0.002*
Diastolic blood pressure, mm Hg	82.9 ± 12.0	80.9 ± 11.3	*0.11*
Hypertension, %	42.1	30.7	*0.02*
Plasma total cholesterol,[e] mmol/l	5.7 ± 1.1	5.6 ± 1.1	*0.29*
Plasma HDL-cholesterol,[e] mmol/l	1.3 ± 0.4	1.3 ± 0.3	*0.23*
Hypercholesterolemia, %	28.5	20.1	*0.04*

[a] Values are means ± SD, unless indicated otherwise.
[b] Controls were matched on age, gender, and enrollment date.
[c] Paired t-test for linear values Wilcoxon signed-rank test for proportions.
[d] Completed higher vocational training or university.
[e] Nonfasting.

Plasma linoleic acid was inversely correlated with plasma arachidonic acid (r=-0.27). Alpha-linolenic acid was positively correlated with EPA-DHA (r=0.29). Alpha-linolenic acid was inversely correlated with linoleic acid (-0.19) (all $P<0.001$).

Table 8.2 shows fatty acid levels for stroke cases and matched controls. The n-6 PUFA linoleic acid and arachidonic acid contributed ~55% and ~6.5% respectively to total fatty acids in cholesteryl esters. N-3 PUFA levels alpha-linolenic acid contributed ~0.5% and EPA-DHA ~1.3%, with an EPA to DHA ratio of ~3:2. Fatty acid levels did not differ between cases and controls, except for EPA-DHA which was higher in cases of total stroke ($P=0.07$) and cases of ischemic stroke ($P=0.02$). Of note, the standard deviation for EPA-DHA in cases was relatively large compared to controls.

In **Table 8.3**, ORs (95% CI) of incident total stroke and stroke subtypes are presented per SD increase (based on the distribution of controls) in the n-6 and n-3 fatty acids. After adjustment for confounders, n-6 PUFA were not associated with incident stroke. The ORs (95% CI) for total stroke were 0.95 (0.74-1.23) per SD increase of linoleic acid and 1.02 (0.80-1.30) per SD increase of arachidonic acid. N-3 fatty acids were also not related to total stroke risk, with ORs (95% CI) for total stroke of 0.94 (0.72-1.21) for alpha-linolenic acid and 1.16 (0.94-1.45) for EPA plus DHA. In addition, no significant associations were observed between n-6 and n-3 fatty acid status and incidence of ischemic or hemorrhagic stroke.

Table 8.2 Fatty acid proportions in plasma cholesteryl esters in 179 Dutch stroke cases and 179 matched controls[a,b]

Fatty acids (g/100g)[c]		Cases	Controls	*P-value*[d]
Total stroke		*n=179*	*n=179*	
Linoleic acid	C18:2n-6	54.4 ± 5.8	55.2 ± 5.3	*0.17*
Arachidonic acid	C20:4n-6	6.6 ± 1.7	6.5 ± 1.6	*0.70*
Alpha-linolenic acid	C18:3n-3	0.53 ± 0.14	0.52 ± 0.15	*0.80*
EPA-DHA	C20:5n-3 + C22:6n-3	1.43 ± 1.04	1.23 ± 0.56	*0.07*[e]
Ischemic stroke		*n=93*	*n=93*	
Linoleic acid	C18:2n-6	54.2 ± 5.7	55.4 ± 5.5	*0.15*
Arachidonic acid	C20:4n-6	6.7 ± 1.7	6.4 ± 1.5	*0.15*
Alpha-linolenic acid	C18:3n-3	0.53 ± 0.13	0.52 ± 0.14	*0.41*
EPA-DHA	C20:5n-3 + C22:6n-3	1.57 ± 1.25	1.25 ± 0.60	*0.02*[e]
Hemorrhagic stroke		*n=50*	*n=50*	
Linoleic acid	C18:2n-6	53.5 ± 6.1	55.2 ± 4.6	*0.14*
Arachidonic acid	C20:4n-6	6.4 ± 1.7	6.6 ± 1.7	*0.49*
Alpha-linolenic acid	C18:3n-3	0.54 ± 0.14	0.54 ± 0.16	*0.86*
EPA-DHA	C20:5n-3 + C22:6n-3	1.29 ± 0.78	1.12 ± 0.40	*0.45*[e]

[a] Fatty acid levels are expressed as mass percentages of total fatty acids.
[b] Controls were matched on age, gender, and enrollment date.
[c] Fatty acid levels are expressed as means ±SD.
[d] Paired t-test.
[e] Paired t-test was performed on the log transformed values of EPA-DHA.

Table 8.3 Associations between plasma fatty acids and incident stroke, matched by age, gender, and enrollment date[a]

	Model 1[b] OR (95% CI)	Model 2[c] OR (95% CI)
Total stroke	*n=179*	*n=179*
Linoleic acid	0.87 (0.71-1.07)	0.95 (0.74-1.24)
Arachidonic acid	1.04 (0.84-1.29)	1.02 (0.80-1.30)
Alpha-linolenic acid	1.03 (0.82-1.29)	0.94 (0.72-1.21)
EPA-DHA	1.23 (1.02-1.50)	1.16 (0.94-1.45)
Ischemic stroke	*n=93*	*n=93*
Linoleic acid	0.79 (0.57-1.09)	0.81 (0.54-1.24)
Arachidonic acid	1.22 (0.93-1.62)	1.21 (0.88-1.67)
Alpha-linolenic acid	1.14 (0.84-1.56)	1.02 (0.71-1.46)
EPA-DHA	1.34 (1.01-1.78)	1.33 (0.96-1.84)
Hemorrhagic stroke	*n=50*	*n=50*
Linoleic acid	0.74 (0.49-1.11)	1.01 (0.56-1.83)
Arachidonic acid	0.87 (0.59-1.29)	0.84 (0.51-1.39)
Alpha-linolenic acid	0.96 (0.62-1.49)	0.73 (0.40-1.32)
EPA-DHA	1.23 (0.90-1.68)	1.08 (0.75-1.57)

Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; OR, odds ratio.

[a] Values are odds ratios (95% CI) per standard deviation increase, based on conditional logistic models.

[b] Crude model, matched for age, gender, and enrollment date.

[c] Additional adjustment for smoking, BMI, education level, alcohol intake, diabetes, hypertension, hypercholesterolemia.

Discussion

The present nested case-control study in a general Dutch population, showed no association between cholesteryl ester plasma levels of n-6 fatty acids (linoleic acid and arachidonic acid) or n-3 (alpha-linolenic acid and EPA-DHA) fatty acids and incidence of total stroke or stroke subtypes.

Several methodological issues should be addressed. The nested case-control design is considered an efficient alternative to a full-cohort analysis.[28] In addition, controls were selected based on incidence density sampling to reduce the likelihood of biased results.[22,23] Cholesteryl ester levels of linoleic acid, alpha-linolenic acid, EPA, and DHA are considered a reliable proxy of dietary intake of n-3 PUFA during the previous weeks.[14,15] because they are not (alpha-linolenic acid and linoleic acid) or hardly (EPA and DHA) endogenously synthesized.[9] In Western diets, arachidonic levels are more influenced by synthesis from linoleic acid than by dietary intake.[7]

The blood samples had been stored for 12-17 years which may have affected the quality of plasma fatty acids. However, storage up to 10 years at -80°C did not significantly influence

serum cholesteryl ester fatty acid profiles in a recent validation study.[29] In addition, the number of detected fatty acids (15-20) and the percentage of unknown fractions (rule of thumb <5 g/100g) were as expected. Furthermore, potential measurement error will have been random because the plasma samples of cases and controls were identically handled and analyzed in random order, and lab technicians were blinded for disease outcome.

Epidemiological studies of n-6 and n-3 fatty acid status and stroke are scarce. In a nested case-control study in 192 American middle-aged men at high risk for cardiovascular diseases in the MRFIT study,[16] the n-6 PUFA linoleic acid or arachidonic acid were not related to stroke risk. This is in line with the results of the present study. In our study, the n-3 PUFA alpha-linolenic acid status were also not associated with incident total stroke or ischemic stroke. In this respect our results differed from those of the MRFIT study, which had a similar distribution of alpha-linolenic acid in serum cholesteryl esters, but a shorter follow-up time of 7 years. In the MRFIT study, a 1-SD higher alpha-linolenic acid level (0.13 g/100g) was associated with a 37% lower risk of total stroke (OR: 0.63; 95% CI: 0.43-0.92).[16]

In the present study, plasma EPA-DHA was unrelated to total stroke incidence, which is in agreement with the MRFIT study[16] and with a Swedish nested case-control study with 169 cases of incident stroke and 738 matched controls.[18] Although results were not statistically significant and confidence intervals were wide, we observed a borderline significant positive association of plasma EPA-DHA with ischemic stroke, but not with hemorrhagic stroke. Also in the Swedish study,[18] a borderline positive association of EPA-DHA in erythrocytes was found with ischemic stroke, but only in men, not in women. Based on these results it is not possible to draw a conclusion on the association between plasma EPA-DHA and stroke incidence.

N-3 PUFA levels are low in cholesteryl esters. Therefore, the variation between persons may have been small compared to the within-subject variability. An American validation study reported that short and long-term reliability coefficients i.e. the ratio of between-person variance to total variance were >0.7 for cholesteryl ester linoleic acid, whereas these coefficients ranged between 0.4-0.5 for fatty acids that composed <1% of total cholesteryl ester fatty acids. The method variability was only <5% of the total variability.[30] A low between to within person variation ratio will hamper finding significant associations between these fatty acids and stroke incidence, although inverse associations of cholesteryl ester alpha-linolenic acid with stroke[16] and EPA-DHA with CHD[31] have been reported previously.

In conclusion, the present study did not find significant associations of plasma n-6 or n-3 fatty acids with incident stroke or stroke subtypes. The number of prospective studies on biomarkers of fatty acid intake and stroke is limited and those available are rather small. Therefore, more and larger prospective studies are needed to establish the relationship between PUFA status and stroke risk.

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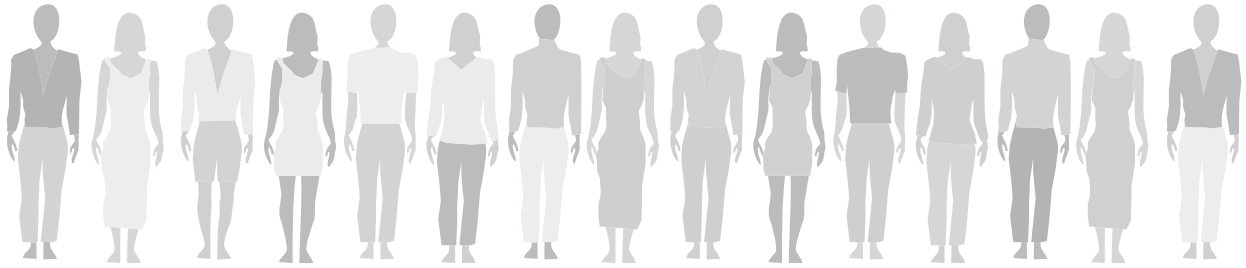

General discussion

9

This thesis addressed the relations of n-6 PUFA (linoleic acid and arachidonic acid) and n-3 PUFA (ALA, EPA, and DHA) with cardiovascular diseases in a general Dutch population. The associations of both dietary intake and plasma levels of these fatty acids with incident CHD and stroke were examined prospectively by means of cohort and nested case-control studies. In addition, we reviewed the recent literature on dietary ALA intake, ALA tissue concentrations, and cardiovascular health in humans (**Chapter 3**).

Main findings

Tables 9.1 and 9.2 provide an overview of the results of this thesis. In **Chapter 2** we concluded that a 4-5 en% difference in linoleic acid intake did not translate into either a different ratio of total to HDL-cholesterol or a different CHD incidence. An ALA intake in quintiles ranging from <1 g/d to >1.9 g/d was also not associated with incident CHD (**Chapter 4**). For EPA-DHA intake, we found an inverse dose-response relation with fatal CHD and fatal MI, but not with nonfatal MI. In the top quartile (~250 mg/d) a ~50% lower risk of fatal CHD and fatal MI was observed compared with the bottom quartile (~40 mg/d). For fish consumption, similar results were observed (**Chapter 5**). With regard to PUFA status, we observed an inverse, but statistically non-significant association between plasma cholesteryl ester linoleic acid and fatal CHD. When we pooled these data with those from similar prospective studies in a meta-analysis, however, a 5% higher cholesteryl ester linoleic acid level was related to a significant 9% lower CHD risk. Cholesteryl ester levels of arachidonic acid and the n-3 PUFA ALA, EPA, and DHA were not associated with CHD risk (**Chapter 7**).

Table 9.1 Main findings on coronary heart disease

Fatty acid	Fatty acid	Intake	Status
n-6 PUFA	Linoleic acid	No association with either the ratio of total to HDL-cholesterol or CHD incidence	Inverse, but non-significant association with fatal MI and sudden cardiac death Meta-analysis: significant inverse association with CHD risk
	Arachidonic acid	Not studied	No association with fatal MI and sudden cardiac death
n-3 PUFA	ALA	No association with incident CHD	No association with fatal MI and sudden cardiac death
	EPA-DHA, fish	Significant inverse association with fatal CHD and fatal MI, not with nonfatal MI	No association with fatal MI and sudden cardiac death

Abbreviations: ALA, alpha-linolenic acid; CHD, coronary heart disease; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; MI, myocardial infarction; PUFA, polyunsaturated fatty acids.

Table 9.2 Main findings on stroke

Fatty acid	Fatty acid	Intake	Status
n-6 PUFA	Linoleic acid	Not studied	No association with incident stroke or subtypes
	Arachidonic acid	Not studied	No association with incident stroke or subtypes
n-3 PUFA	ALA	Borderline significant inverse association with incident stroke, most pronounced for ALA from salad dressings.	No association with incident stroke or subtypes
	EPA-DHA, fish	Significantly inversely associated with incident stroke in women, but not in men	No association with incident stroke or subtypes

Abbreviations: ALA, alpha-linolenic acid; CHD, coronary heart disease; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; MI, myocardial infarction; PUFA, polyunsaturated fatty acids.

An ALA intake ≥1.1 g/d was associated with a 35-50% lower risk of incident stroke, compared with an intake <1.1 g/d (**Chapter 4**). In women, an inverse dose-response relation was observed for EPA-DHA and fish intake with incident stroke, with a ~50% lower risk in the top quartile compared with the bottom quartile. For men, these associations were weaker and not statistically significant (**Chapter 6**). In plasma cholesteryl esters, n-6 or n-3 PUFA were not related to incident stroke (**Chapter 8**).

PUFA intake

In the MORGEN study, we assessed habitual PUFA intake by an FFQ that reflected the intake of the previous year. Fatty acid intakes were prospectively studied in relation to CHD and stroke by means of a prospective cohort design (**Chapter 2, 4-6**).

Assessment of PUFA intake

One of the major methodological problems of fatty acid research is that fat intake is difficult to assess through dietary questionnaires and interviews.[1,2] This leads to misclassification of exposure, which in turn may result in dilution of associations. Fat used for food preparation and added fat such as gravy and sauces are hard to quantify in dietary surveys. Additionally, PUFA such as linoleic acid and ALA are difficult to assess and disentangle, as they are both present in similar products, including margarines and cooking fats.[3] To illustrate this, out of the top-10 products that contributed the most to the linoleic acid intake in our population, seven were also top-10 ALA contributors (unpublished results). Examples of these products are soybean oil, margarine,

mayonnaise, and bread, which are part of commonly consumed diets. Similarly, ALA and linoleic acid in various foods are highly correlated with other fatty acids. An additional source of error is the calculation of PUFA intake with food composition tables. Most epidemiological studies with clinical endpoints use FFQ data. With an FFQ, people can be ranked according to their dietary intake.[4]

PUFA compositions of foods such as margarines and cooking oils have changed over time. Therefore, even if people have used the same brands of cooking oils and margarines during their lifetimes, their fatty acid intakes have changed. In the studies reported in this thesis, diet was assessed between 1993 and 1997, after which participants were followed until 2007. In the nineties, *trans*-fat was removed from margarines and cooking fats. Furthermore, in margarine spreads total fat content has been reduced and the ratio of saturated fatty acids to PUFA has been improved. The fatty acid composition of baseline diets in our study may therefore be less favorable than diets during follow-up. However, the overall fatty acid improvements in margarines and cooking fats probably occurred in the whole population, without necessarily affecting the ranking of individuals for specific PUFA intakes. The impact on our results of the addition of extra ALA in margarines is small, because this occurred only towards the end of our follow-up (after 2003), and only involved some brands.

PUFA intake and CVD

In controlled dietary experiments, dietary PUFA, which are mainly n-6 PUFA, have a favorable effect on plasma LDL-cholesterol, the total to HDL-cholesterol ratio[5,6] and consequently CHD risk.[7,8] However, most individual prospective cohort studies, including ours (**Chapter 2**), have failed to link a higher PUFA intake (or a lower saturated fat intake) to a lower CHD risk.[9] In addition, we did not observe an association between linoleic acid intake and plasma cholesterol levels in a cross-sectional analysis. This is most likely due to the error in the single dietary assessment.[10] The predicted differences in plasma cholesterol based on the population distribution of linoleic acid are modest. The predicted difference for a 4-5 en% higher linoleic acid intake was -0.15 for the ratio of plasma total to HDL cholesterol. This would correspond to a ~7% lower CHD incidence,[7] which is hard to demonstrate in observational prospective cohort studies.[11]

Two meta-analyses of cohort studies recently reported on the effect of a higher PUFA intake as a substitute for saturated fat. The meta-analysis of Jakobsen *et al.*[12] showed that the replacement of 5 en% from saturated fatty acids by PUFA was significantly associated with a 13% lower risk for coronary events. This was, however, not supported by the meta-analysis of Siri-Tarino *et al.*[13] The latter meta-analysis mainly focused on the effects of saturated fat, but also (in sub-analyses) on the exchange of saturated fat by PUFA. The conflicting results of the two meta-analyses may be explained by the different methodologies used. Jakobsen *et al.*[12] pooled study-specific HRs, based on individual data with similar statistical models for all included studies, as opposed to Siri-Tarino *et al*. The studies in the latter meta-analysis may therefore be less comparable and the pooled estimate less precise. Furthermore, Jakobsen *et al.*[12] selected studies on basis of quality

of intake data, which may have decreased misclassification of exposure of the included studies in comparison with the studies included by Siri-Tarino *et al*.[13] The large Nurses' Health Study in which PUFA were inversely associated with CHD will have largely influenced the overall results of the meta-analyses. Jakobsen *et al*.[12] included the results of the Nurses' Health Study[14] twice, by dividing the 14-year follow-up into two periods to take advantage of the repeated assessments of dietary intake (in 1980 and 1984) and the long follow-up. However, these sub-cohorts are not independent and the results of the nurses may contribute too much to the overall pooled estimate of Jakobsen *et al*.[12] Siri-Tarino *et al*.[13] used the most recent publication of the Nurses' Health Study of Oh *et al*.[15] with 20-years of follow-up. These two meta-analyses of prospective cohort studies showed different results on the effect of replacement of saturated fatty acids by PUFA. However, in a meta-analysis of eight RCTs, replacement of 5 en% of SFA by PUFA reduced coronary events by 10%.[16] A contrast of only 5 en% in a prospective cohort study with a single assessment of PUFA intake may be too small to demonstrate a CHD benefit, due to measurement error.

In **Chapter 4**, we found no evidence that ALA intake within the narrow range of our population, i.e. quintiles ranging from <1.0 g/d to >1.9 g/d, was associated with incident CHD. In addition, we analyzed different ALA sources in relation to CHD and stroke, i.e. ALA from salad dressings (mayonnaise and soybean oil, which were the main ALA sources) and other ALA sources. Both ALA groups, however, were not associated with incident CHD. Our results were in line with the Zutphen Study in elderly Dutch men, with a range of ALA intake similar to our study.[17] In contrast to our study, *trans*-fatty acid intake for the men in the Zutphen Study who were examined in 1985 was still quite high.[18] In that study, ALA intake from sources also containing *trans*-fatty acids was positively associated with CHD, whereas ALA from sources without *trans*-fatty acids was not associated with CHD.[17] Results on ALA and CHD from observational cohort studies are inconsistent. For example, in the Nurses' Health Study, ALA was inversely associated with sudden cardiac death, but not with nonfatal MI.[19,20] In the Health Professionals Follow-up Study, ALA was inversely associated with incident MI,[21] nonfatal MI,[22] and incident CHD,[22] but not with sudden cardiac death[22] or fatal CHD.[22] In a large case-control study from Costa Rica, ALA intake was inversely associated with nonfatal MI. The ALA intake in Costa Rica ranged from 1.1 g/d in the bottom decile to 2.4 g/d in the top decile. The population distribution of ALA intake in Costa Rica was higher than in the Netherlands. Despite that the results from Costa Rica suggested that benefits of ALA on CHD could already be achieved at modest levels (~1.8 g/d),[23,24] this was, however, not supported by our prospective data.

We found an inverse association of ALA intake with incident total and ischemic stroke, which was most pronounced for ALA from salad dressings (**Chapter 4**). Stratification on sources is potentially an interesting way to remove residual confounding by saturated fatty acids, present in for example margarines, but to a lesser extent in salad dressings. We adjusted for many potential confounders, including the intake of raw vegetables, which were also inversely associated with incident stroke in the same cohort.[25] However, the associations may still be influenced by a healthier diet and lifestyle of those who regularly eat (raw) vegetables or salads. The fact that we found an inverse association of ALA intake with incident stroke but not with incident CHD

was surprising. Adjustment for systolic blood pressure, which is stronger related to stroke than to CHD, did not change our results. This raises the hypothesis that ALA has specific neurological effects.[26-30] To the best of our knowledge, our cohort study was the first to show an inverse association of ALA intake with stroke. Confirmation of our findings in other prospective studies is warranted, before drawing conclusions.

Despite potential measurement error and misclassification, we observed significant inverse associations of fish and EPA-DHA intake with both fatal CHD (**Chapter 5**) and stroke (**Chapter 6**). In the MORGEN study, 40% of the population consumed fish less than once per month and 30% at least once a week, which created a contrast in the distribution of intake of the population. Our results on CHD are in agreement with a large body of evidence from prospective cohort studies, indicating a cardio-protective effect for low doses (~250 mg/d) of omega-3 fatty acids in fish.[31] In cohort studies, residual confounding by other lifestyle habits is always a concern, which can be overcome in double-blind RCTs. At present, the best estimate of risk reduction based on observational studies and trials of the association of modest fish consumption (1-2 fish meals/week) on CHD is 36%.[31]

In women, we observed a significant inverse association of EPA-DHA with incident stroke, whereas for men the association was inverse, but much weaker and not statistically significant. Our findings were similar to most other observational studies in Western countries, as described in **Chapter 6**. We could not explain the gender difference by different EPA-DHA distributions or different stroke types. It could be speculated that women are more aware of what is purchased and used for cooking. They may therefore report their diets more accurately than men, resulting in less misclassification of exposure. This is supported by validation studies of the used FFQ, in which reproducibility and validity for fish intake were higher for women compared to men.[32] The different associations for men and women with regard to dietary intake and cardiovascular disease endpoints deserve further study.

Conclusions for PUFA intake

The lack of an inverse association of linoleic acid with CHD in prospective cohort studies may be due to the limited range of distributions of intake in combination with measurement errors. Also for ALA, similar limitations applied, yet the total evidence for an inverse association of ALA intake with CHD is less convincing than for linoleic acid. The inverse association of ALA intake with incident stroke is of interest and may be biologically plausible. This therefore deserves further attention in other studies. The inverse relation of EPA-DHA with fatal CHD is supported by a large body of evidence from prospective cohort studies indicating a cardio-protective effect for low doses of EPA-DHA intake (~1 fish meal per week). In women, EPA-DHA was significantly inversely associated with stroke, whereas in men only a weak non-significant inverse association was present. Gender differences in observational nutritional epidemiology have received little attention and deserve further study.

Plasma PUFA

We measured plasma cholesteryl ester PUFA in the Monitoring Project on Cardiovascular Disease Risk Factors (MP-CVDRF) and the MORGEN study, which are two Dutch population-based cohorts that were examined in different time periods (1987-1991 and 1993-1997, respectively). Fatty acid levels were studied in relation to fatal CHD (**Chapter 7**) and incident stroke (**Chapter 8**) with a nested case-control design. For CHD, we used both cohort studies, whereas for stroke we only used the MORGEN study, as nonfatal stroke was not assessed in MP-CVDRF.

Assessment of plasma PUFA

Fatty acid status can be measured as free fatty acids in serum (or plasma), as components of triglycerides, phospholipids, cholesteryl esters, erythrocytes, platelets or in adipose tissue from various sites.[1] The amount of specific fatty acids varies between tissue fractions (**Figure 9.1**). For the assessment of PUFA status, we used plasma cholesteryl esters, a fraction that is relatively stable over time if stored at -80°C.[33] Cholesteryl esters and phospholipids are more or less comparable with regard to the time frame of intake. Alternatively, erythrocytes could have been used, which reflect intake of the previous months. However, erythrocytes are relatively

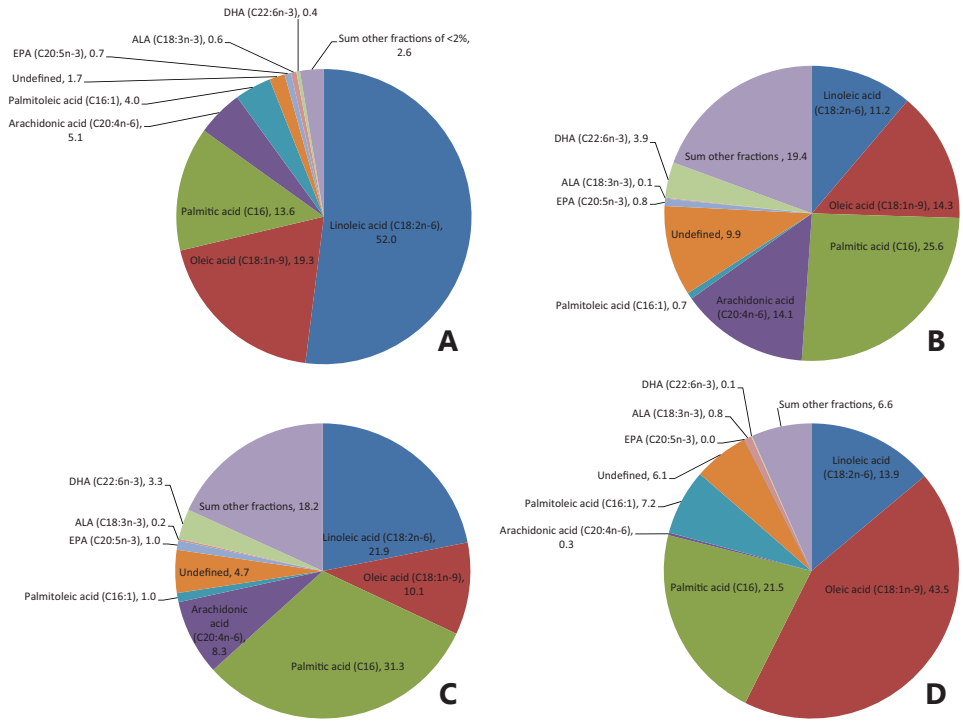

Figure 9.1 Fatty acid profiles in cholesteryl esters (A) , erythrocytes (B), phospholipids (C), and adipose tissue (D) (adapted from Hodson *et al.*[35]).

sensitive to oxidation, because of hemoglobin-derived iron. Furthermore, erythrocytes require specific handling and storage conditions.

The samples of MP-CVDRF were stored for 18-23 years at -30°C and the samples of the MORGEN study were stored for 12-17 years at -80°C. Storage up to 10 years at -80°C did not significantly influence serum cholesteryl ester fatty acid profiles in a recent validation study.[33] Salo *et al*.[34] observed that despite marked alterations in the fatty acid composition after three years of storage at -20°C, the results of the two repeated measurements correlated highly with each other. Additionally, for linoleic acid, ALA, and EPA, plasma cholesteryl esters performed better than plasma phospholipids or plasma triglycerides, especially for ALA. Salo *et al*.[34] also reported that the relative decrease in PUFA proportions after three years of storage at -20°C was positively correlated with the number of double bonds of the PUFA. Unfortunately, like most prospective cohort studies, we do not have data on the specific effects of storage temperature and time on PUFA levels in our population, but probably oxidation of cholesteryl ester PUFA occurred, especially for the samples of MP-CVDRF that were stored at -30°C. The mean levels of the n-3 and n-6 PUFA were all lower in MP-CVDRF than in the MORGEN study (**Chapter 7**; **Figure 9.2**).

Of concern is, however, whether the degree of oxidation was higher for samples with originally higher PUFA levels. This could have resulted in a different ranking of individuals as compared with the situation at baseline. That could in turn have distorted an association of higher PUFA levels with a lower risk of CHD or stroke, if it existed. The percentage of unknown fractions was higher for MP-CVDRF as compared with the MORGEN study, whereas for both cohorts, the percentage of unknown fractions did not differ between cases and controls. Furthermore, the ratio of palmitic acid to arachidonic acid (the largest SFA and PUFA contributors in cholesteryl esters), did also not differ between cases and controls, whereas this ratio was higher in the MP-CVDRF compared to the MORGEN study. This suggests that a potential decrease in PUFA levels was probably non-differential between cases of CHD or stroke and healthy controls in our nested cases-control studies.

In cholesteryl esters, linoleic acid, oleic acid, palmitic acid, and arachidonic acid together represent ~80% of the fatty acid spectrum and linoleic acid is by far the most important fatty acid fraction. This was also the case for our samples of MP-CVDRF and the MORGEN study (**Figure 9.2**). Besides by the diet, the spectrum is largely determined by the specificity of the enzyme lecithin, which has a preference for linoleic acid.[36,37] **Figure 9.1A and 9.2** also clearly show that n-3 PUFA are very low in cholesteryl esters (less than 1% of total fatty acids). For our samples, the analytical measurement error of the cholesteryl ester PUFA was small. Coefficients of variation of the quality control plasma pool, analyzed in duplicate in each run, ranged between 3 and 3.5% (intra and inter assay combined). Laboratory technicians did not know which samples belonged to the cases and which to the controls. Furthermore, cases and controls were randomly distributed over the runs. An American validation study on cholesteryl esters reported, that short

and long-term reliability coefficients, i.e. the ratio of between-person variation to total variation, were >0.7 for linoleic acid, whereas these coefficients ranged between 0.4-0.5 for fatty acids that composed <1% of total fatty acids. The variation of the method contributed for only <5% to the total variation, which means that the measurement itself was highly reproducible.[38] A low between to within-person variation ratio will hamper finding significant associations between the studied fatty acids and fatal CHD.

Plasma PUFA and CVD

As discussed in previous chapters, none of the cholesteryl ester n-6 or n-3 PUFA was statistically significantly related to incident stroke (**Chapter 8**) or fatal CHD (**Chapter 7**). The few other published studies on plasma PUFA and stroke used different fractions and results were inconsistent.[39-41] In **Chapter 7**, we additionally conducted a meta-analysis of prospective cohort studies that specifically used cholesteryl esters. In that meta-analysis, a 5% higher linoleic acid level, which was equal to ~1 standard deviation in the MORGEN study, was significantly inversely associated with a 9% lower CHD risk. The other fatty acids were not associated with CHD risk in the meta-analysis.

In 2007, Harris *et al.*[42] conducted a meta-analysis of 25 (nested) case-control and prospective cohort studies on tissue fatty acid composition and risk of CHD. For the subgroup of prospective studies, DHA, ALA (borderline statistically significant), but not EPA, were inversely associated with fatal CHD. Several differences in design could be responsible for the different results between their and our meta-analysis. Although Harris *et al.*[42] combined data of a large number of studies, 16 of the 25 studies had a classical case-control design (based on prevalent cases), which is more prone to reverse-causation and selection bias. Seven studies (case-control studies only) were based on adipose tissue samples. The other 18 used various blood fractions, such as phospholipids, cholesteryl esters, and erythrocytes, which could cause substantial heterogeneity in the meta-analysis results. Finally, the analysis was based on crude PUFA levels. Potential confounding e.g. by body mass index and smoking, which appeared to be confounders in our nested case-control study, may partly explain discrepant results between the two meta-analyses.

Conclusions for plasma PUFA

The inverse association of plasma linoleic acid with CHD risk in our meta-analysis supports the hypothesis that linoleic acid protects against CHD. Plasma arachidonic acid and n-3 PUFA were not related to fatal CHD. No associations of plasma n-3 and n-6 PUFA with incident stroke were found. Should inverse associations of cholesteryl ester PUFA with CHD or stroke exist, then they will be hard to demonstrate in individual studies on PUFA status, if the variation between persons is small compared to the within-person variation.

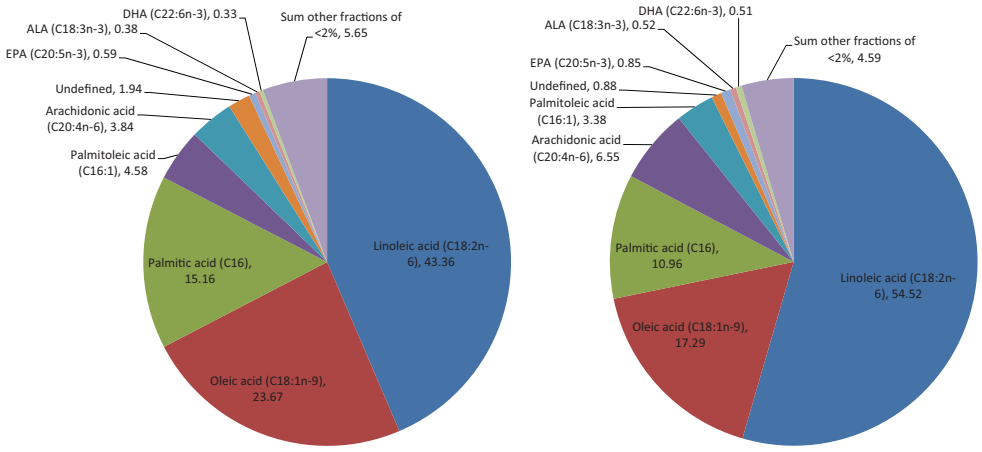

Figure 9.2 Cholesteryl ester fatty acid profiles in MP-CVDRF (left) and the MORGEN study (right).

PUFA and cardiovascular diseases: intake or status?

As shown in **Table 9.1 and 9.2**, our results on PUFA intake and PUFA status differed. The results on plasma EPA-DHA (**Chapter 7 and 8**) did not support our results on EPA-DHA intake on fatal CHD (**Chapter 5**) and incident stroke (**Chapter 6**). Similarly, our results on ALA intake in relation to incident stroke (**Chapter 3**) were not supported by plasma ALA data (**Chapter 8**).

Biomarkers of intake

Fatty acid levels in human tissue may provide a more accurate measure of habitual intake than dietary records or questionnaire data, especially when the nutrient of interest varies widely in foods and food groups and when food composition tables are inaccurate for that specific nutrient.[43] Furthermore, tissue levels are not dependent on a person's ability to recall dietary intakes. Because linoleic acid and ALA are essential fatty acids, their concentration in tissue is expected to reflect dietary intake to some extent. This also applies to EPA-DHA, for which endogenous synthesis is limited.[44-46] To overcome difficulties with subjective dietary assessment, PUFA status is often used as a biomarker of intake in epidemiological studies.[47,48] Fatty acid status reflects the combination of fatty acid intake and metabolism in the body and is therefore a more proximal measure between exposure and disease.

Despite advantages of biomarkers of intake compared to dietary assessment methods, there are also disadvantages. PUFA status may be influenced by metabolic differences between people with regard to genetic background, background diet, gender, BMI, smoking etc. Additional error

is introduced by day-to-day variation in tissue levels and exchange of fatty acids with other body pools. Furthermore, error is introduced by differences in tissue sampling handling, and storage, and by the laboratory measurement.[49]

Correlations between PUFA intake and plasma PUFA

The average compositions of fatty acid intake and plasma cholesteryl ester fatty acids in the MORGEN study clearly differ (**Figure 9.3**). For the MORGEN study, we assessed the correlation coefficients between intake and status (n=457 participants; unpublished results). Cholesteryl ester linoleic acid, EPA, and DHA correlated reasonably well with intake whereas plasma ALA was only weakly correlated with intake (**Table 9.3**).

It is difficult to compare correlation coefficient between studies, because studies differ in populations, in distributions of intake, and in the fractions and dietary assessment methods that are being compared. Furthermore, intake can be expressed in absolute terms (g/d), relative to total energy intake, or relative to total fat intake. The time frames of intake and status information are also important. Additionally, in an experimental study, large contrasts in intake of specific fatty acids can be achieved, whereas in an observational cohort study intake of several fatty acids differ between people and the range of intake is often small.

In our data, plasma values of PUFA indicated intake over the previous weeks, whereas FFQ data indicated intake over longer time periods (months to year), which will have affected the correlation between these two measures. Despite the different reference periods, correlation

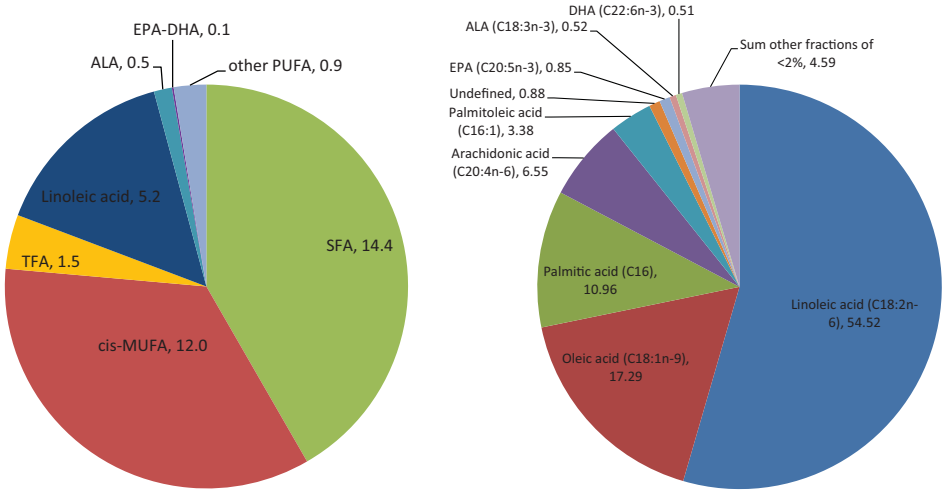

Figure 9.3 Dietary fatty acids (% of total energy intake; left) and cholesteryl ester fatty acids in the MORGEN study (right).

Table 9.3 Correlations between cholesteryl ester PUFA and dietary PUFA (n=457)[a]

		Linoleic acid	ALA	EPA	DHA
Linoleic acid	Plasma	–			
	Absolute intake	0.28[b]	**0.75**[c]	**0.05**	**0.11**
	Intake/total energy	0.34			
	Intake/energy from fat	0.30			
ALA	Plasma cholesteryl ester	*-0.19*[d]	–		
	Absolute intake		0.06	**0.05**	**0.11**
	Intake/total energy		0.12		
	Intake/energy from fat		0.12		
EPA	Plasma cholesteryl ester	*-0.53*	*0.30*	–	
	Absolute intake			0.34	**0.95**
	Intake/total energy			0.36	
	Intake/energy from fat			0.37	
DHA	Plasma cholesteryl ester	*-0.17*	*0.07*	*0.63*	–
	Absolute intake				0.40
	Intake/total energy				0.43
	Intake/energy from fat				0.43

[a] Spearman correlation coefficients.
[b] Correlation coefficients between intake and plasma PUFA are depicted in colored boxes.
[c] Correlation coefficients between intake of different PUFA are depicted in bold font.
[d] Correlation coefficients between plasma PUFA are depicted in italic font.

coefficients were 0.30-0.40 for linoleic acid, EPA, and DHA, In a review by Hodson *et al.*,[35] correlation coefficients for PUFA in blood fractions compared with FFQ data of 8-9 studies largely varied (**Table 9.4**) Limited data are available for correlations between cholesteryl ester and FFQ PUFA, however, our correlation coefficients seemed reasonable compared to other validation studies.

In a Dutch validation study, a correlation coefficient based on the comparison of adipose tissue with 19 24h recalls of 0.70 was reported for linoleic acid. The median correlation for the individual recalls was 0.28, which shows the dilution due to large day-to-day variation in fatty acid intake.[50] Compared to the above mentioned extensive validation study on fatty acids, correlation coefficients between dietary and plasma PUFA of 0.30 are generally regarded as reasonable to good. However, a coefficient of 0.30 only explains <10% of the total variance. The remaining 90% of variance is explained by other factors, such as variation between people with reporting PUFA intake and metabolism. Therefore, differences between associations of intake and plasma PUFA with CVD endpoints could be expected.

Table 9.4 Overview of correlation studies for PUFA intake with PUFA status (adapted from Hodson *et al.*[35])

Author	Subjects	Blood fraction	Dietary assessment	Fatty acid									
				14:0	15:0	16:0	18:0	18:1 n-9	18:2 n-6	18:3 n-3	20:4 n-6	20:5 n-3	22:6 n-3
Baylin *et al.*, 2005, Am J Epidemiol	196 M and F	Wb Total	FFQ[e]		**0.23**[a]	0.14	0.03	**0.19**	**0.43**	**0.38**	0.05	**0.22**	**0.23**
Sarkkinen *et al.*, 1994, Am J Clin Nutr	160 M and F	plt PL	5 x 3 DDR[e]			**0.15**[a]			**0.54**				
Sarkkinen *et al.*, 1994, Am J Clin Nutr	160 M and F	rbc PL	5 x 3 DDR[e]			0.04[a]			**0.55**				
Feunekes *et al.*, 1993, Am J Clin Nutr	99 M and F	rbc PL	FFQ[e]						**0.44**[a]				
			DH[e]						**0.41**				
Romon *et al.*, 1995, Metabolism	244 M	rbc PL	3 DDR[e]						**0.64**[a]				
Sun *et al.*, 2007, Am J Clin Nutr	306 F	rbc PL	FFQ[e]	**0.16**[a]		0.03	0.01	**0.14**	**0.24**	**0.18**	-0.04	**0.38**	**0.56**
Van Houwelingen *et al.*, 1989, Eur J Clin Nutr	61 M	p Total	DH[i]						**0.59**[a]			**0.41**	**0.51**
Andersen *et al.*, 1999, Am J Epidemiol	125 M	p Total	FFQ[e]			0.11[a]		0.09	0.16	**0.28**		**0.51**	**0.52**
Kuriki *et al.*, 2003, J Nutr	79 F	p Total	7 DDR[e]	0.18[b]		0.10	0.14	**0.30**	0.16	**0.24**	0.03	**0.69**	**0.59**
Sun *et al.*, 2007, Am J Clin Nutr	306 F	p Total	FFQ[e]	**0.20**[a]		**0.12**	0.06	**0.12**	**0.25**	**0.23**	-0.01	**0.21**	**0.48**
Baylin *et al.*, 2005, Am J Epidemiol	196 M and F	p Total	FFQ[e]		**0.26**[a]	0.14	0.01	**0.21**	**0.41**	**0.39**	0.12	**0.28**	**0.31**
Astorg *et al.*, 2007, Eur J Clin Nutr	276 M	p Total	15 x 24 h recall[f]						**0.22**[a]	0.06	**0.16**	**0.24**	**0.25**
Van Houwelingen *et al.*, 1989, Eur J Clin Nutr	61 M	p TAG	DH[i]						**0.72**[a]			**0.33**	**0.49**
James *et al.*, 1993, Am J Clin Nutr	30 M	p TAG	7 x 3 DDR[h]						**0.77**[a]				
Asciutti-Moura *et al.*, 1988, Am J Clin Nutr	53 M and F	p TAG	7 DDR[e]					0.19[a]	**0.44**				
Van Houwelingen *et al.*, 1989, Eur J Clin Nutr	61 M	p CE	DH[i]						**0.67**[a]			**0.27**	**0.30**
James *et al.*, 1993, Am J Clin Nutr	30 M	p CE	7 x 3 DDR[h]						**0.70**[a]				
Sarkkinen *et al.*, 1994, Am J Clin Nutr	160 M and F	p CE	5 x 3 DDR[e]			**0.34**			**0.49**				
Ma *et al.*, 1995, Am J Clin Nutr	3570 M and F	p CE	FFQ[e]			**0.19**[a]			**0.28**	**0.21**		**0.23**	**0.42**
Wolk *et al.*, 2001, J Nutr	114 M	p CE	2 x 7 DDR[e]	**0.33**[a]	**0.30**			0.21[a]	0.23				
Asciutti-Moura *et al.*, 1988, Am J Clin Nutr	53 M and F	p CE	7 DDR[e]										

Table 9.4 continues on next page

Table 9.4 *Continued*

Author	Subjects	Blood fraction	Dietary assessment	Fatty acid									
				14:0	15:0	16:0	18:0	18:1 n-9	18:2 n-6	18:3 n-3	20:4 n-6	20:5 n-3	22:6 n-3
Van Houwelingen *et al.*, 1989, Eur J Clin Nutr	61 M	p PL	DH[i]						**0.49**[a]			**0.35**	**0.41**
Andersen *et al.*, 1996, Am J Clin Nutr	579 M and F	p PL	FFQ[g]			**-0.23**[c]		**-0.21**	0.01	-0.04		**0.51**	**0.49**
James *et al.*, 1993, Am J Clin Nutr	30 M	p PL	7 x 3 DDR[h]						0.44[a]				
Wolk *et al.*, 2001, J Nutr	114 M	p PL	2 x 7 DDR[e]	**0.33**[a]	**0.30**			0.13[a]	**0.29**				
Asciutti-Moura *et al.*, 1988, Am J Clin Nutr	53 M and F	p PL	7 DDR[e]										
Hodge *et al.*, 2007, Nutr Metab Cardiovasc Dis	4439 M and F	p PL	FFQ[e]			0.17[ad]		0.45[ad]	0.58[d]	0.24[d]		0.40[d]	0.78[d]
Asciutti-Moura *et al.*, 1988, Am J Clin Nutr	53 M and F	p NEFA	7 DDR[e]					0.09[a]	0.18				

Abbreviations: M, males; F, females; total, total fatty acids; TAG, triacylglycerol; DH, dietary history; DDR, day diet record; FFQ, food frequency questionnaire; wb, whole blood; rbc, red blood cells; p, plasma; CE, cholesteryl ester; PL, total phospholipid; NEFA, non-esterified fatty acids; plt, platelet.

Correlation coefficients in bold indicate statistical significance ($P < 0.05$) as reported by paper.

Fatty acids expressed as

[a] weight %

[b] mol%

[c] µmol/L

[d] Statistical significance not reported

Dietary variable expressed as

[e] % of total fat intake

[f] % of total energy intake

[g] g/kg body weight

[h] g/day

[i] not stated

Conclusions and implications

Within the range of intake of this generally healthy Dutch population, linoleic acid intake was not associated with either a different ratio of total to HDL-cholesterol or a different CHD incidence. However, the inverse association of plasma linoleic acid with CHD risk in our meta-analysis supported the hypothesis that linoleic acid protects against CHD. Both dietary and plasma ALA were not associated with CHD. Although the overall intake was low, we still had sufficient contrast in EPA-DHA and fish intake to find an inverse relation with fatal CHD and fatal MI. In contrast, the range of intake did not result in sufficient contrast in plasma EPA-DHA values, which did therefore not confirm the inverse associations of EPA-DHA intake with fatal CHD. ALA and, in women, EPA-DHA (and fish) intake were inversely associated with the risk of incident stroke. However, no associations were observed between plasma PUFA and stroke.

PUFA intake and plasma PUFA profiles are very different. Data on dietary intake and plasma values have their own specific drawbacks, and associations of intake and status with CHD or stroke are therefore not necessarily similar. Additionally, fatty acid profiles also considerably differ between tissue fractions. The lack of associations that were expected to be present raises concern regarding the validity of PUFA assessed from cholesteryl ester or other fractions as a biomarker of intake for epidemiological studies on PUFA. This does not imply that biomarkers of PUFA intake cannot be very useful as compliance marker in trials.

For both intake and status data, the lack of inverse associations of PUFA intake or status with CHD or stroke in prospective cohort studies may be due to the limited range of variation between people in the population in combination with measurement error. Should inverse associations of PUFA with CHD or stroke exist, then they will be hard to demonstrate in individual studies if the variation between persons is small compared to the within-person variation. Within populations, a single FFQ or a single plasma measurement will therefore often be too limited to link PUFA exposure to CVD endpoints. Biomarkers can be an alternative, however, they are not a solution to measurement error of intake.

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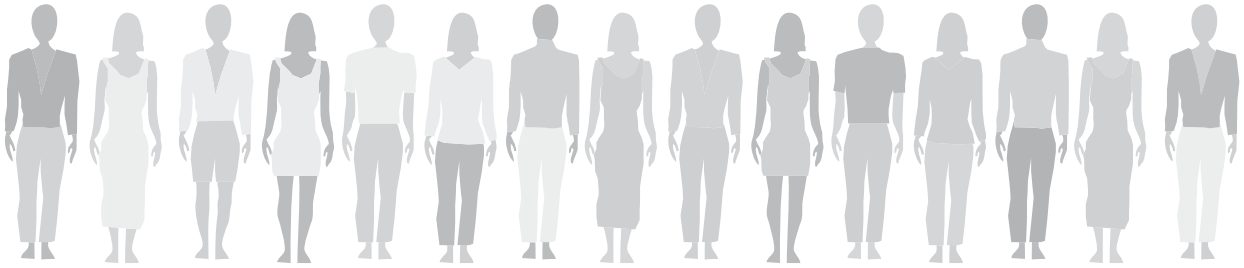


Summary in Dutch (Samenvatting)

Dit proefschrift richt zich op *cis*-meervoudig-onverzadigde vetzuren in de voeding. Deze vetzuren, met meer dan één dubbele binding, worden verder ingedeeld als n-6 (of omega-6) en n-3 (of omega-3) vetzuren. N-6 vetzuren dragen voor 85-90% bij aan de totale inname van meervoudig-onverzadigde vetzuren en n-3 vetzuren leveren de overige 10-15%. Het n-6 vetzuur linolzuur is het meest voorkomende meervoudig-onverzadigde vetzuur in de voeding en heeft als belangrijke bronnen plantaardige oliën, zoals zonnebloemolie en sojaolie. Het is een 'essentieel' vetzuur, wat betekent dat de mens het niet zelf kan aanmaken. Alfa-linoleenzuur is een essentieel n-3 vetzuur uit plantaardige bronnen. Het komt vooral voor in sojaolie, canolaolie, lijnzaadolie en walnoten. Eicosapentaeenzuur (EPA) en docosahexaeenzuur (DHA) zijn n-3 vetzuren die veel voorkomen in vis. Het menselijk lichaam kán alfa-linoleenzuur omzetten in EPA en vervolgens DHA, maar de opbrengst hiervan is laag.

Hoewel er de afgelopen tientallen jaren al veel onderzoek naar is gedaan, is de rol van meervoudig-onverzadigde vetzuren in relatie tot het voorkómen van coronaire hartziekten en beroerte nog steeds onderwerp van discussie. Inconsistente resultaten uit epidemiologische studies komen mogelijk deels door methodologische beperkingen van (subjectieve) voedingsvragenlijsten. Dit probleem zou vermeden kunnen worden door vetzuurniveaus in bloedplasma te meten als objectieve indicator ("biomarker") voor de inname van vetzuren. In dit proefschrift worden inname en plasmaniveaus van verschillende n-3 en n-6 vetzuren onderzocht in relatie tot coronaire hartziekten en beroerte in de Nederlandse situatie.

We hebben gegevens gebruikt van ruim 20.000 mannen en vrouwen in de leeftijd van 20 tot 65 jaar die aan het begin van het onderzoek geen hart- en vaatziekten hadden. De gegevens zijn afkomstig van het MORGEN-cohort van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) te Bilthoven. MORGEN staat voor "MOnitoring van Risicofactoren en Gezondheid in Nederland". In het MORGEN-cohort is het vóórkomen van leefstijl- en risicofactoren gemeten bij een steekproef van inwoners van Amsterdam, Doetinchem en Maastricht. Tussen 1993 en 1997 zijn alle deelnemers onderzocht. Daarna werd bijgehouden welke mensen in het ziekenhuis werden opgenomen of overleden. Hiervoor is gebruik gemaakt van de Landelijke Medische Registratie (LMR) van ziekenhuisopnames en de doodsoorzakenstatistieken van het Centraal Bureau voor de Statistiek. De mensen, van wie de gebruikelijke voedselconsumptie per persoon was nagevraagd met een vragenlijst over 178 voedingsitems, werden tien jaar gevolgd. Gedurende die tijd werd geregistreerd wie van de deelnemers hart- en vaatziekten kregen. De samenhang tussen de inname van de verschillende vetzuren en het optreden van coronaire hartziekten en beroerte werd onderzocht met behulp van cohort studies. In geneste case-controle studies werd de relatie tussen vetzuurniveaus in het bloed met coronaire hartziekten en beroerte bestudeerd.

In **hoofdstuk 2** hebben we onderzocht of een hogere inname van linolzuur, uitgewisseld met een isocalorisch lagere inname van koolhydraten, gerelateerd was aan een lager risico op coronaire hartziekten. De belangrijkste voedingsbronnen van linolzuur waren margarine

(21%), oliën (13%), brood (12%), noten (10%), varkensvlees (10%) en sauzen (9%). Gedurende de onderzoeksperiode van 10 jaar kregen 280 van de 20069 mannen en vrouwen coronaire hartziekten. Diabetespatiënten en deelnemers die medicijnen gebruikten tegen een hoge bloeddruk of een hoog cholesterol waren uitgesloten van deze studie. De inname van linolzuur, ingedeeld in 5 gelijke groepen (quintielen), liep op van 3.6 tot 8.0 procent ten opzichte van de totale energie-inname. Bij vrouwen, maar niet bij mannen, hing een hogere linolzuurinname samen met lagere bloedwaarden van totaal cholesterol en HDL-cholesterol. Er was geen relatie tussen linolzuur met de ratio van totaal/HDL-cholesterol en ook niet met de kans om coronaire hartziekten te krijgen. De hazard ratio's van de quintielen waren niet significant verschillend van het laagste quintiel van linolzuurinname en varieerden tussen 0.83 en 1.00. Onze conclusie was dat een verschil van 4-5 energieprocent van de inname van linolzuur of koolhydraten zich niet vertaalde in een andere totaal/HDL-cholesterolratio of een ander risico op coronaire hartziekten.

Hoofdstuk 3 beschrijft de actuele literatuur (gepubliceerd vanaf 2008) over alfa-linoleenzuur in relatie tot cardiovasculaire risicofactoren en ziekten bij mensen. Als er extra alfa-linoleenzuur wordt ingenomen, stijgt het niveau ervan in het bloed. Echter, de effecten op ziekterisico zijn nog onduidelijk. Het wachten is op langere-termijn interventiestudies waarmee het individuele effect van alfa-linoleenzuur op hart- en vaatziektenrisico bestudeerd kan worden.

In **hoofdstuk 4** hebben we gekeken of een hogere alfa-linoleenzuurinname resulteerde in een kleinere kans op het optreden van coronaire hartziekten en beroertes. De belangrijkste voedingsbronnen van alfa-linoleenzuur waren mayonaise (15%), margarine (14%), sojaolie (8%) en brood (8%). Tijdens de onderzoeksperiode kregen 280 mensen een coronaire hartziekte en 221 mensen een beroerte. De inname van alfa-linoleenzuur varieerde van minder dan 1.0 g/d in het laagste quintiel tot meer dan 1.9 g/d in het hoogste quintiel. Alfa-linoleenzuur bleek niet samen te hangen met het risico op coronaire hartziekten, met hazard ratio's (quintielen 2-5) die varieerden tussen 0.89 en 1.01 zonder dat deze significant verschilden van de groep mensen met de laagste 20% van de inname. Echter, ten opzichte van deze laagste 20%, had de rest van de onderzoeksgroep een 35-50% lager risico op een beroerte. In aanvulling op bovenstaande resultaten hebben we alfa-linoleenzuur uit dressings (mayonaise en sojaolie), waar weinig verzadigd of *transvet* in zit, apart onderzocht van alfa-linoleenzuur uit andere bronnen. Dit omdat alfa-linoleenzuur in veel voedingsmiddelen sterk positief gecorreleerd is met gehaltes van verzadigd en *transvet*, en het daardoor lastig is om de effecten van elkaar te scheiden. Het bleek dat de gevonden inverse associatie met beroerte wél duidelijk zichtbaar was voor alfa-linoleenzuur uit dressings, maar niet voor alfa-linoleenzuur uit andere bronnen. Met coronaire hartziekten zagen we opnieuw geen associatie. Onze conclusie was dat een lage inname van alfa-linoleenzuur een risicofactor zou kunnen zijn voor een beroerte, maar dat dit wel eerst moet worden bevestigd in andere epidemiologische studies en interventiestudies.

In Nederland wordt weinig vis gegeten. In **hoofdstuk 5** hebben we onderzocht of er binnen de steekproef uit de Nederlandse bevolking toch een associatie te zien was van EPA-DHA en vis met het optreden van coronaire hartziekten. In de onderzoeksperiode overleden 647 van de 21.342 personen, van wie 82 aan coronaire hartziekten. Verder overleefden 252 personen een hartinfarct. Het risico op sterfte aan een coronaire hartziekte was 49% lager bij personen die relatief veel visvetzuren (meer dan 194 milligram per dag) binnenkregen, dan bij mensen met weinig visvetzuren in hun voeding (minder dan 62 milligram per dag). Als er specifiek gekeken werd naar het hartinfarct, was het risico zelfs 62% lager. Een hartinfarct zonder dodelijke afloop hing niet samen met de visvetzuren. Vergelijkbare resultaten werden gevonden als de mensen werden ingedeeld op hun visconsumptie, overeenkomend met meer dan 14 gram vis per dag ten opzichte van minder dan 3,3 gram. De resultaten sluiten aan bij ander wetenschappelijk onderzoek waarbij visvetzuren vooral lijken te beschermen tegen levensbedreigende ritmestoornissen, die vaak een rol spelen bij een dodelijk hartinfarct. Een kleine hoeveelheid visvetzuren (EPA en DHA) in de voeding zou dus al het risico kunnen verminderen op het risico om te overlijden aan een hartziekte, met name aan een hartinfarct.

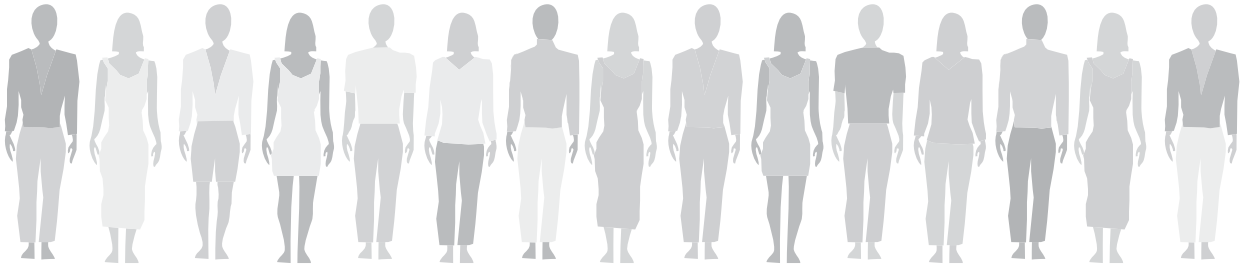
In de literatuur zijn er aanwijzingen dat de mogelijke bescherming van EPA-DHA en vis tegen beroerte bij vrouwen sterker is dan bij mannen. Dit werd bestudeerd in **hoofdstuk 6**. De meeste van de 221 beroertes (n=142) waren het gevolg van een bloedstolsel in de bloedvaten van de hersenen (inclusief 60 transiente ischemische attacks; TIA's). Verder waren er 47 het gevolg van een hersenbloeding. Van 32 beroertes was de oorzaak niet bekend. Bij vrouwen bleek het risico op een beroerte 51% lager te zijn bij de hoogste 25% van de visvetzuurinname vergeleken met de laagste 25%. Bij mannen leek EPA-DHA ook gunstig te zijn, maar de resultaten waren veel minder duidelijk en zeker niet statistisch significant. Vergelijkbare resultaten werden gevonden voor visconsumptie. We konden de verschillende resultaten tussen mannen en vrouwen niet verklaren door een andere inname van EPA-DHA of vis, of door verschillen in de typen beroerte. In de literatuur zijn er ook geen aanwijzingen dat het effect van EPA-DHA of vis op beroerte bij vrouwen anders is dan bij mannen. Bij vervolgonderzoek is het dus met name belangrijk om erachter te komen of er een fysiologische verklaring is voor de verschillende resultaten tussen de geslachten.

In **hoofdstuk 7** hebben we onderzocht of n-6 en n-3 vetzuren gemeten in plasma cholesteryl esters (een onderdeel van bloed) gerelateerd waren aan sterfte aan coronaire hartziekten. Voor deze studie hebben we gegevens van het MORGEN-cohort en van het 'Peilstationsproject' (een iets oudere, maar vergelijkbare steekproef van het RIVM) gecombineerd. De deelnemers waren 8-19 jaar gevolgd voor het optreden van ziekte. Voor elk van de 279 deelnemers die overleden aan een coronaire hartziekte werd een vergelijkbaar, ander persoon geselecteerd van hetzelfde geslacht en dezelfde leeftijd, die tot dat moment niet was overleden. De patiënten en de geselecteerde gezonde mensen kwamen in ons geneste case-controle onderzoek terecht en we bepaalden bij hen vetzuurniveaus in het bloed. Een hoger niveau van linolzuur hing samen met een lager risico op een fatale hartziekte, maar dit was niet statistisch significant. Echter, als

we de resultaten van vergelijkbare andere prospectieve epidemiologische studies combineerden in een meta-analyse, vonden we dat een 5% hoger plasmaniveau van linolzuur overeenkwam met een 9% lager risico op coronaire hartziekten. Arachidonzuur, alfa-linoleenzuur, EPA en DHA waren niet gerelateerd aan coronaire hartziekten.

In **hoofdstuk 8** deden we een vergelijkbaar genest case-controle onderzoek, maar dan voor beroerte. We gebruikten hiervoor alleen het MORGEN-cohort. Uitgaande van de 179 mensen die een beroerte hadden gekregen werd een steekproef getrokken van 179 vergelijkbare deelnemers zonder beroerte. De vetzuurniveaus van linolzuur, arachidonzuur, alfa-linoleenzuur, EPA en DHA in bloedplasma bleken niet gerelateerd aan het risico op een beroerte.

De belangrijkste bevindingen, methodologische aspecten en de interpretatie van de resultaten worden beschreven in **hoofdstuk 9**. Onze hypothese dat linolzuur een gunstig effect zou hebben op het risico op coronaire hartziekten werd bevestigd in onze biomarker studie, maar niet als we de linolzuurinname gebaseerd op de voedingsvragenlijst analyseerden. Voor EPA-DHA vonden we juist een gunstig effect op coronaire hartziekten en beroerte met behulp van de voedingsinformatie maar niet op basis van de vetzuurniveaus in het bloed. Hetzelfde gold voor alfa-linoleenzuur in relatie tot beroerte. Mogelijk zijn de discrepanties tussen de bevindingen (deels) te wijten aan de beperkte verschillen binnen de Nederlandse bevolking in combinatie met de meetfouten de inherent zijn aan de gebruikte methoden om vetzuurinname te meten.



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De AOT zal ik nooit vergeten. Super dat ik daar onderdeel van mocht zijn. In onze strak geplande tweewekelijkse "EC" vergaderingen was er meestal veel te bespreken, maar de sfeer was altijd erg goed. Ook bij het Alpha Omega Coördinatie Centrum was het altijd gezellig en er was vaak wel iets te beleven. Ik denk dat veel collega's jaloers waren op ons team. Ook aan de verschillende AOT-uitjes denk ik met veel plezier terug, met voor mij als persoonlijke topper de rondvaart en het palingroken in Belt-Schutsloot. Hierbij wil ik graag naast Marianne en Daan de andere vaste AOT-teamleden bedanken: Annemarie, Erik, Marianne Pluigers, Linda, Janny, Lucy en Eveline.

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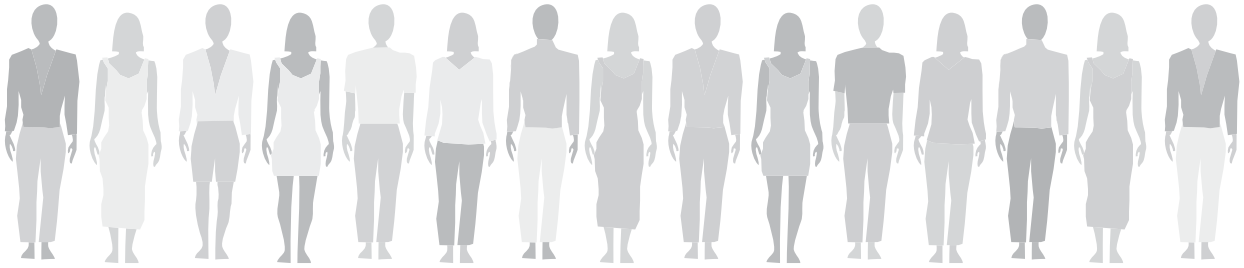

About the author

Curriculum vitae

Janette de Goede was born on the 22nd of November 1978 in Zwolle, the Netherlands. In 1996, she started to study Human Nutrition at Wageningen University. She performed an MSc thesis project at the department of Human and Animal Physiology of Wageningen University, entitled 'The effect of natural flavonoids at the thyroid metabolism under circumstances of marginal iodide deficiency in rats'. Her second MSc thesis and internship was performed at the Medical Research Council in Cape Town, South Africa, where she worked on the project 'Primary prevention of non-communicable diseases by community health workers: a case study of health and development.' In between these two MSc projects she worked as a research assistant for the FACIT-Study, a large intervention study on the influence of folic acid on atherosclerosis at the Division of Human Nutrition, Wageningen University. After her MSc graduation in 2001 she was again involved in the FACIT-study until December 2001. From 2002-2010 she worked as a trial manager for the Alpha Omega Trial, a randomized, placebo-controlled, double blind multi-centre study to examine the effect of low doses of eicosapentaenoic acid and docosahexaenoic acid and alpha-linolenic acid on cardiovascular mortality in 4837 post-MI patients. Her main responsibilities were the daily supervision of the research team, communication with participants, obtaining medical ethical approvals, coordination and maintenance of the Alpha Omega database, logistics of blood samples and measurements, randomization, and monitoring of patients.

In July 2006, she started her PhD research on the intake of polyunsaturated fatty acids and cardiovascular diseases, of which results are described in this thesis. For this PhD project she performed epidemiological data analyses at the National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands. Furthermore, she continued working for the Alpha Omega Trial until the trial was completed in 2010. Janette joined several (international) conferences, followed courses on epidemiology and statistics, supervised 10 BSc and MSc students, and participated in various committees and discussion groups of the Division of Human Nutrition. In 2006 she was selected to participate in the '10-day teaching seminar on cardiovascular disease' of the World Heart Federation and in 2011 she was selected for the European Nutritional Leadership Program. Since August 2011 she has been appointed at the Netherlands Nutrition Center, The Hague.

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Original research papers

Goede J de, Verschuren WMM, Boer JMA, Kromhout D, Geleijnse JM. Gender-specific associations of marine n-3 fatty acids and fish consumption with 10-year incidence of stroke. Submitted.

Goede J de, Verschuren WMM, Boer JMA, Verberne LDM, Kromhout D, Geleijnse JM. N-6 and n-3 fatty acid cholesteryl esters in relation to fatal coronary heart disease in a Dutch adult population: a nested case-control study. Submitted.

Goede J de, Verschuren WMM, Boer JMA, Kromhout D, Geleijnse JM. N-6 and n-3 fatty acid cholesteryl esters in relation to incident stroke in a Dutch adult population: a nested case-control study. Submitted.

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Educational program

Discipline specific activities

Courses

- Nutritional and lifestyle epidemiology, VLAG, Wageningen (NL), 2003
- Master class Geriatric Nutrition, VLAG, Wageningen (NL), 2004
- 10-day teaching seminar cardiovascular disease epidemiology, World Heart Federation, Mount Tamborine (Australia), 2006
- Concepts and methods in epidemiology, Division of Human Nutrition, Wageningen (NL), 2006/2007
- Principles of epidemiologic data analysis, NIHES, Lunteren (NL), 2007
- Regression analysis, NIHES, Rotterdam (NL), 2007
- Survival analysis, NIHES, Rotterdam (NL), 2007
- Multi-level analysis, RIVM, Bilthoven (NL), 2010
- Linear and logistic regression, Division of Human Nutrition, Wageningen (NL), 2010

Meetings

- Science meetings Netherlands Heart Foundation (NL), Leiden/Amsterdam, 2004, 2005, 2008
- NWO Nutrition meetings, Arnhem/Deurne (NL), 2004, 2005, 2008, 2010
- Symposium "Omega-3: Uitdaging voor voeding en technologie", NVVL, Ede (NL), 2005
- Annual meetings of the Netherlands Epidemiology Society (WEON) (NL), Wageningen, 2005 (NL), Maastricht, 2007 (NL), Amsterdam 2009 (NL)
- Master class Nutrition Communication: challenges and opportunities, VLAG, Wageningen (NL), 2005
- Prospective registration of trials in the Netherlands, Dutch Cochrane Centre, Amsterdam (NL), 2005
- European Congress of Epidemiology: "Epidemiology and Health Care Practice", Utrecht (NL), 2006
- Nutrition and Ageing Symposium, VLAG, Wageningen (NL), 2006
- Master class Dietary Influences on blood pressure, VLAG / NZO, Wageningen (NL), 2006
- 1st Nutrition and Health Congress, Amsterdam (NL), 2006
- Lipids and Brain: "PUFA metabolism, function and protection against diseases", EFECG/DGF, Paris (France), 2007
- Symposium "N-3 fatty acids and the brain", VLAG, Wageningen (NL), 2007
- Borrelpraatsessie "Twee keer per week vis, haalbaar voor mens en vis?", NVVL Schiedam (NL), 2008
- EPIC NL Symposium, UMCU, Utrecht (NL), 2008
- Wageningen Nutritional Sciences Forum, Division of Human Nutrition, Arnhem (NL), 2009

- Annual Conference on Cardiovascular Disease Epidemiology and Prevention, American Heart Association, Palm Harbor, Florida (USA), 2009 and San Francisco, California (USA), 2010
- "Alpha-linolenic acid and cardiovascular diseases", Product Board for Margarine, Fats and Oils, Rijswijk (NL), 2009
- 9th Unilever Nutrition Symposium "Essential fats for future health", Unilever R&D, Vlaardingen (NL), 2010
- Congress of the International Society for the Study of Fatty Acids and Lipids, Maastricht (NL), 2010
- "Eet voor je leven", Voeding Nederland, Ede (NL), 2010
- Congress of the European Society of Cardiology, Stockholm (Sweden), 2010

General courses

- Good Clinical Practice, ICH Training en Advies, Utrecht (NL), 2004
- Scientific oral presentation skills, Wageningen Centre for Food Sciences, Wageningen (NL), 2004
- Personal communication development, Strategic Development Group Oisterwijk, Wageningen (NL), 2005
- Organizing and supervising student projects, OWU, Wageningen (NL), 2005
- Competence Assessment, Wageningen Graduate School, Wageningen (NL), 2006
- VLAG PhD week, VLAG, Bilthoven (NL), 2007
- Scientific Writing, WUR/CENTA, Wageningen (NL), 2008
- NWO Talent Class: Media training, NWO, The Hague (NL), 2008
- NWO Talent Day: Creative Thinking, Social Networks and Career, Utrecht (NL), 2010
- Career Perspectives, Wageningen Graduate School, Wageningen (NL), 2010
- European Nutrition Leadership Programme (ENLP), Luxemburg, 2011

Optional courses and activities

- Preparation PhD research proposal, Wageningen (NL), 2006
- Literature meeting "Journal club", Division of Human Nutrition, Wageningen (NL), 2006
- Literature meeting "N-3 club", Division of Human Nutrition, Wageningen (NL), 2004-2006
- PhD study tour (USA), member of organizing committee, Division of Human Nutrition, Wageningen (NL), 2007
- Literature meeting "Oldsmobiles", Division of Human Nutrition, Wageningen (NL), 2006/2011
- Research meetings, Division of Human Nutrition, Wageningen (NL), 2006/2011
- Literature group "CVD club", Division of Human Nutrition, Wageningen (NL), 2008/2009
- PhD study tour Denmark, Sweden, Finland, Division of Human Nutrition, Wageningen, 2009

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