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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.

Abbreviations: CI, confidence interval; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MORGEN, Monitoring Project on Risk Factors for Chronic Diseases; OR, odds ratio; PUFA, polyunsaturated fatty acids; SD, standard deviation.

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Introduction

Worldwide, stroke is the second largest cause of death and a major cause of long-term disability [1,2]. Stroke was the third leading 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 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 22,654 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 who did not provide informed consent for vital status follow-up, participants without dietary information, and 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 non-fasting blood at baseline. EDTA-plasma was stored at −80 °C until analyzed in 2010. Fatty

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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/100 g). 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. [25]. Body weight, height, and blood pressure were measured at baseline by trained research nurses. Hypertension was defined as a systolic blood pressure $\geq$140 mmHg, a diastolic blood pressure $\geq$90 mmHg, or the use of blood pressure lowering medication. Non-fasting plasma was analyzed for total and high-density lipoprotein (HDL) cholesterol, and hypercholesterolemia was defined as plasma total cholesterol $\geq$ 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, 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 $\leq$ 2 and women $\leq$ 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 ($\pm$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), presence of diabetes mellitus (yes/no), hypertension (yes/no), hypercholesterolemia (yes/no). Two-sided *p*-values $\leq$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 less educated, had higher blood pressures, and more often had hypercholesterolemia or diabetes mellitus (Table 1). 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 2 shows fatty acid levels for stroke cases and matched controls. The n-6 PUFA linoleic acid and arachidonic acid contributed $\sim$55% and $\sim$6.5% respectively to total fatty acids in cholesteryl esters. N-3 PUFA levels alpha-linolenic acid contributed $\sim$0.5% and EPA-DHA $\sim$1.3%, with an EPA to DHA ratio of $\sim$3:2. Fatty acid levels did not differ between cases and controls, except for EPA-DHA which was higher in cases of total stroke ($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 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.

Discussion

The present nested case—control study in an adult 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

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Table 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, %			
No intake	20	11	–
Low to 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, mmol/l[e]	5.7 ± 1.1	5.6 ± 1.1	*0.29*
Plasma HDL-cholesterol, mmol/l[e]	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] Non-fasting.

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/100 g) were as expected. Furthermore, potential measurement error will

Table 2 Fatty acid proportions in plasma cholesteryl esters in 179 Dutch stroke cases and 179 matched controls.[a,b]

Fatty acids (g/100 g)[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

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Table 3 Associations between plasma fatty acids and incident stroke, matched by age, gender, and enrollment date[a]

	Model 1[b]	Model 2[c]
	OR (95% CI)	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)

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

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 was 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/100 g) 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.

Fish intake in the Netherlands is low. In the full cohort, 40% of the population consumed fish less than once per month, 8.5% never consumed fish, and 30% consumed fish at least once a week [30]. This means that the contrast in fish consumption in our study was small. Despite the low fish intake, we observed correlation coefficients of ~0.4 for EPA and DHA in cholesteryl esters vs. intake data ($n = 457$ participants of the MORGEN study; unpublished results) which seemed reasonable compared to other validation studies [31].

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 and 0.5 for fatty acids that composed <1% of total cholesteryl ester fatty acids. The method variability was only <5% of the total variability [32]. 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 [33] 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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Disclosures

None.

Author contributions

J.G., J.M.G., and D.K. designed the study; J.M.A.B. and W.M.M.V. provided the data and critically reviewed the manuscript; J.G. analyzed the data and performed

statistical analyses; J.G. drafted the paper; J.G., J.M.G., and D.K. had primary responsibility for final content. All authors read and approved the final version of the manuscript.

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