Title page

Title:

Intake of alpha-linolenic acid is not consistently associated with a lower risk of peripheral artery disease: results from a Danish cohort study

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Abstract

Intake of the plant-derived omega-3 fatty acid alpha-linolenic acid (ALA) has been associated with anti-atherosclerotic properties. However, information on the association between ALA intake and development of peripheral artery disease (PAD) is lacking. In this follow-up study, we investigated the association between dietary intake of ALA and the rate of PAD among middle-aged Danish men and women enrolled into the Danish Diet, Cancer and Health cohort between 1993 and 1997. Incident PAD cases were identified through the Danish National Patient Register. Intake of ALA was assessed using a validated food frequency questionnaire. Statistical analyses were performed using Cox proportional hazard regression allowing for separate baseline hazards among sexes and adjusted for established risk factors for PAD. During a median of 13.6 years of follow-up, we identified 950 valid cases of PAD with complete information on covariates. The median energy-adjusted ALA intake within the cohort was 1.76 g/d (95% central range: 0.94-3.28). In multivariable analyses, we found no statistically significant association between intake of ALA and the rate of PAD (P = 0.339). Also, no statistically significant associations were observed in analyses including additional adjustment for co-morbidities and in sex-specific analyses. In supplemental analyses with additional adjustment for potential dietary risk factors, we found a weak inverse association to PAD with ALA intake above the median, but the association was not statistically significant (P = 0.314). In conclusion, dietary intake of ALA was not consistently associated with decreased risk of PAD.

Introduction

Peripheral artery disease (PAD) in the lower extremities is a chronic atherosclerotic disease characterised by stenosis and occlusion of the arteries, covering a clinical spectrum from no symptoms to effort-induced ischemic muscle discomfort and/or pain and critical ischemia with tissue loss[1–3]. Symptomatic PAD is associated with functional limitations, diminished quality of life and a high risk for major cardiovascular events and death[4,5]. The global burden of PAD is expected to increase markedly in the near future and identification of factors that may lower disease risk is urged[6].

The plant-derived n-3 fatty acid alpha-linolenic acid (ALA) is a precursor of long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFAs), which may influence inflammatory processes that may be involved in development and progression of atherosclerosis[7–9]. However, the conversion capacity of ALA into LC n-3 may be limited in humans[10]. ALA may possibly exert health benefits independent of its precursor role[11] and has been suggested to be an important nutrient in the Mediterranean diet[12], which has been ascribed many health benefits including lowering risk of PAD[13] and major cardiovascular events[14].

The majority of previous follow-up studies investigating the association between ALA intake and the risk of atherosclerotic cardiovascular disease have focused on coronary heart disease (CHD). Some cohort studies have reported inverse associations between ALA intake and CHD risk[15–20], but the results have not been consistent[18,21–27]. Few studies have investigated associations between ALA intake and the risk of ischemic stroke[22,23,28–30], but to our knowledge no previous follow-up studies have investigated the association between ALA intake and the risk of PAD.

The objective of this study was to investigate the association between intake of ALA and the risk of PAD. We hypothesized that intake of ALA would be inversely associated with the risk of incident PAD.

Methods

Study population and design

This follow-up study was based on data from the Diet, Cancer and Health cohort that was established to investigate the role of diet and lifestyle in relation to cancer and other chronic diseases[31]. The recruitment procedures, sample size considerations and collection of data have been described in detail elsewhere[31]. Briefly, native citizens aged 50-64 years who were living in and

around Copenhagen and Aarhus in Denmark without a previous diagnosis of cancer were invited between 1993 and 1997 to participate in the study. Potential eligible participants were identified through the Danish Civil Registration System in which every citizen living in Denmark is provided with a unique identification number[31].

The Diet, Cancer and Health cohort study was conducted according to the guidelines laid down in the Declaration of Helsinki and approved by the Health Research Ethics, Capital Region of Denmark, and the Danish Data Protection Agency. All participants gave written informed consent at inclusion[31].

In the present follow-up study, participants registered with a diagnosis of cancer not registered in the Danish Cancer Registry at the time of invitation due to processing delay as well as participants registered with a diagnosis of PAD or chronic kidney disease before enrolment were excluded. Also, participants for whom information on exposure and/or covariates was missing were excluded. The current study has been approved by the Danish Data Protection Agency (2008-58-0028-2016-229).

Exposure assessment

Information on habitual diet over the past 12 months was assessed at baseline using a 192-item semi-quantitative food frequency questionnaire (FFQ)[32]. The average consumption of foods and beverages was reported within 12 categories ranging from never to eight or more times per day. The reported intakes were used to estimate daily intake of ALA and total energy based on Danish food composition tables (version 1.3.2) with the use of the software program FoodCalc (Center for Applied Computer Science, University of Copenhagen; www.ibt.ku.dk/jesper/foodcalc). The FFQ used has previously been validated against two 7-day diet weighed records and found useful for categorizing individuals according to their intake of energy intake and polyunsaturated fat[33]. The intake of ALA was expressed as energy-adjusted intake in g/d using the residual method[34].

Covariates

Detailed information on social, lifestyle and health aspects such as length of schooling, smoking habits, physical activity, history of hypercholesterolemia, hypertension and diabetes, and use of lipid-lowering or anti-hypertensive agents and insulin was collected at baseline using a self-administered questionnaire. The questionnaire was processed by optical scanning and checked for reading errors and omissions at the two study centres where also anthropometric measurements

including height, weight and waist circumference were obtained at enrolment. Information on alcohol consumption, total energy intake and intake of other nutrients was obtained from the FFQ.

Outcome assessment

Incident cases of PAD were identified through record linkage with the Danish National Patient Register, which includes information on outpatient visits and discharge diagnoses from all hospitals in Denmark[35,36].

Potential cases of PAD included participants registered with either a primary or secondary discharge diagnosis of PAD according to the International Classification of Diseases (ICD) codes (ICD-8: 44390; 44500; 44509; 44590; 44599; 44020 and 44030, and ICD-10: I70.2, I70.2A and I73.9A-C). Subsequently, all potential cases of PAD were validated by review of medical records[37]. A registered diagnosis of PAD was considered valid in patients with an ankle brachial index (ABI) < 0.9 and/or a toe brachial index (TBI) < 0.7 and/or demonstration of radiologically significant stenosis or calcifications in relevant arteries in the lower extremities. Patients with an ABI > 1.4 together with a history of diabetes mellitus or treatment by chronic renal dialysis were also considered valid PAD cases. Furthermore, patients that experienced a blood pressure drop in the lower extremities of more than 30mmHg or a 20% drop of the resting ABI immediately after a treadmill test and patients who underwent relevant vascular surgery for atherosclerosis were included as cases. Finally, patients who qualified for vascular surgery for atherosclerosis although not performed due to severe comorbidity were included as cases as well[37].

Participants were followed from baseline until first registration of PAD, death, emigration or end of follow-up in December 2009.

Statistics

Hazard ratios (HRs) were used to describe associations between energy-adjusted ALA intake and the rate of PAD. HRs with 95% confidence intervals (CIs) were calculated using Cox proportional hazard regression with age as underlying timescale allowing for separate baseline hazards among men and women.

Dietary intake of ALA was analysed as a continuous exposure variable using restricted cubic splines with 3 knots with the median intake as reference in order to visualize the shape of the observed associations. The knots were placed at the 10th, 50th and 90th percentile as recommended by Harrell[38]. The spline curves were formally tested against a horizontal line using Wald tests. The

spline plots were restricted to the 95% central range and presented with 95% CIs. Also, analyses with ALA intake divided into quintiles were conducted using the lowest quintile as reference.

We investigated the association between ALA intake and rate of PAD in three different models specified prior to data analysis. In model 1A, we included baseline age (continuous, years) in order to ensure comparison of participants for whom reported exposure information was of the same age. In model 1B, we additionally included information on established risk factors for PAD including: length of schooling (≤7, 8-10, or >10 years), smoking (never, former, current 1-14, 15-24, or >24 g/d), physical activity (inactive, moderately inactive, moderately active, or active), waist-circumference (continuous, cm), body mass index (continuous, kg/m^2) and alcohol intake (continuous, g/d). In model 2, we included the following co-morbidities, which may be considered potential intermediate variables: self-reported history of hypercholesterolemia and/or use of lipid-lowering medication (yes, no, or unknown), hypertension and/or use of anti-hypertensive medication (yes, no, or unknown) and diabetes mellitus and/or use of insulin.
The proportional hazard assumption was evaluated by plotting the scaled Schoenfeld residuals against age at event.

In sensitivity analyses, we plotted the whole exposure range and examined whether the spline curves were robust when the number and location of the knots were modified. Also, additional adjustment for a history of myocardial infarction, ischemic stroke or both prior to enrolment was undertaken as sensitivity analyses.

In supplemental analyses, we investigated the association between ALA intake and the rate of PAD in sex-specific analyses. Also, analyses including adjustment for established risk factors (model 1B) and hormone substitution were undertaken. In further supplemental analyses, potential dietary risk factors were added to model 1B including total energy intake without contribution from alcohol intake (continuous, kJ/d), intake of fiber (continuous, g/d), glycemic load (continuous, unit-less), and intakes of saturated fatty acids, monounsaturated fatty acids, linoleic acid and marine LC n-3 PUFAs (continuous, g/d) (model 3). All continuous covariates were entered into the models using restricted cubic splines with 5 knots.

Data were analyzed using Stata statistical software (version 15; StataCorp LP, US), and a p-value <0.05 was considered statistically significant.

Results

A total of 160,725 men and women were invited to participate in the Diet Cancer and Health cohort study and 57,053 accepted. We excluded 1,805 participants because they had a diagnosis of cancer (n = 569), PAD (n = 330) or chronic kidney disease (n = 31) before enrolment, or for whom information on exposure or other covariates was missing (n = 960) (Figure 1). Among the remaining 55,248 participants, we identified 950 participants that developed PAD during a median of 13.6 (95% central range: 4.3-15.3) years of follow-up. The incidence rate of PAD was 1.32 per 1000 person years.

Baseline characteristics of the participants with complete information on covariates in the cohort and participants that developed PAD during follow-up are shown in Table 1.

The median energy-adjusted dietary ALA intake in the cohort was 1.76 g/d (95% central range: 0.94-3.28).

In spline analyses including adjustment for sex and age (model 1A), we found a positive association between ALA intake and the rate of PAD (Supplemental Figure 1). However, in multivariable analyses including additional adjustment for established risk factors for PAD (model 1B) a weak statistically non-significant inverse U-shaped association between ALA intake and the rate of PAD was observed (P-value = 0.339) (Figure 2). Additional adjustment for co-morbidities (model 2) also showed a weak inverse U-shaped association between ALA intake and the rate of PAD that was not statistically significant (P = 0.338) (Supplemental Figure 2). In supplemental analyses, with additional adjustment for dietary risk factors (model 3), we found a weak inverse association to PAD above the median ALA intake, but the overall association was not statistically significant different from a horizontal line (P-value = 0.314) (Supplemental Figure 3).

Sensitivity analyses indicated that models with ALA intake modelled as restricted cubic splines were robust when the location and number of knots for the exposure of interest were modified.

Categorical analyses of the association between ALA intake in quintiles and the rate of PAD are shown in Table 2. We found similar patterns of associations in analyses of ALA intake in quintiles and the rate of PAD as in the spline analyses. The individual hazards in the second to fifth quintile were not statistically significant different from the reference in the first quintile in either of the multivariable adjusted models. In supplemental analyses, additional adjustment for myocardial infarction and/or ischemic stroke before enrolment did not influence the observed associations.

Also, additional adjustment for use of hormone substitution did not influence the observed associations (data not shown).

Similar patterns of associations between ALA intake and the rate of PAD were observed when the analyses were conducted separately among men and women (Supplemental Table 2).

No evidence of a departure from the proportionality assumption was observed in either of the models (data not shown).

Discussion

In this large follow-up study, we found indications of a weak inverse U-shaped association between ALA intake and the rate of PAD in analyses including adjustment for established risk factors and indications of a weak inverse association between ALA intake and the rate of PAD above the median intake in analyses including adjustment for established risk factors and dietary risk factors. However, none of these associations were statistically significant. Given the relatively weak and statistically non-significant associations observed, this study suggests that dietary intake of ALA is not appreciably associated with the risk of PAD within this population of middle-aged Danish men and women. It should be stressed that this study did not investigate the potential effect of a Mediterranean diet on PAD risk, but the results may indicate that the possible protective effect provided by the Mediterranean dietary pattern on PAD and major cardiovascular events is unlikely to be ascribed to ALA intake.

This study had some limitations that should be mentioned. Participants were followed by linkage with nationwide registries with very limited loss to follow-up, which limits the potential of selection bias. Information on ALA intake was obtained using a self-administered FFQ and measurement error is inevitable, but because of the temporality in a follow-up study, exposure measurement error probably occurred at random, which generally leads to an underestimation of the true association and loss of statistical power. The FFQ used in this study was not specifically developed to assess ALA intake. Further, information on diet was only available at baseline and changes in dietary habits during follow-up may have occurred. Thus, repeated dietary measurements would have been preferable to limit random measurement error and to capture potential changes in dietary habits over time. However, diets of individuals tend to be relatively consistent over intervals of several years[39]. Information bias is unlikely to have influenced the observed associations significantly because diagnoses of PAD were established and validated independently of the dietary assessment.

Identification of PAD cases in this study relied on registered discharge diagnosis of PAD and the

vast majority of identified cases were symptomatic patients. PAD may be underdiagnosed in the general population[40] and cases either asymptomatic or not referred to hospitals were not included in this study. However, random misclassification of a diagnosis of PAD may bias associations towards no association, but because PAD risk in general was relatively low, such potential bias was probably minor. We included detailed information on established risk factors for PAD in the analyses, but residual confounding from known or unknown PAD risk factors may still be of importance for the observed associations. The observed association between ALA intake and the rate of PAD (model 1A) was weakened after adjustment for established risk factors for PAD (model 1B). However, additional adjustment for history of hypercholesterolemia, hypertension and diabetes (model 2) showed a similar pattern of association compared to model 1B, which may indicate that potential residual confounding from these co-morbidities was not of major importance. Notably, these co-morbidities may also be considered intermediates and conditioning for these covariates could potentially introduce collider stratification bias[39], which may bias associations in either direction. In analyses including adjustment for established PAD risk factors and dietary factors (model 3) that may influence PAD risk, the observed association between ALA intake and the rate of PAD was slightly lower compared to model 1B at higher ALA intakes and residual confounding from dietary factors cannot be excluded. However, adjustment for dietary factors may introduce restrictions in the underlying dietary pattern that are not comparable with the ordinary dietary pattern and analyses with and without dietary covariates should not be directly compared. Therefore, given the interpretational challenges of model 2 and 3, we consider model 1B the most appropriate for interpretation.

The Diet, Cancer and Health cohort only included native Danish participants from selected areas in Denmark who had survived until enrolment into the study without a previous diagnosis of cancer, chronic kidney disease or PAD, which may limit the generalizability of the study results.

We decided to conduct the main analyses as sex-stratified analyses by allowing baseline hazards among men and women to differ[5,6]. Thus, the HRs from these analyses should be interpreted as a weighted average of the association in men and women. Previous studies have suggested that the endogenous conversion efficiency of ALA into LC n-3 PUFAs may be stimulated by sex hormones and is greater in women[10]. However, in sex-specific analyses we found similar patterns of association between ALA intake and the risk of PAD among men and women. Almost 60% of the female participants were post-menopausal at baseline[21] and a potential higher conversion efficiency of ALA mediated by female sex hormones may therefore not be of major importance in this cohort.

In supplemental analyses, additional adjustment for hormone substitution at baseline also did not influence the observed associations.

A previous cross-sectional study including 422 cases reported that ALA intake was associated with a lower odds of PAD[41]. Another cross-sectional study including 199 cases reported that the content of ALA in red blood cells was associated with lower odds of lower limb disease[42], whereas two case-control studies did not find any appreciably nor statistically significant differences between circulating levels of ALA between cases and controls[43,44]. However, none of these studies included detailed adjustment for risk factors of PAD and the results should be interpreted with caution due to the risk of residual confounding and reverse causation (cross-sectional studies).

We used restricted cubic splines to evaluate the shape of the association between ALA intake and the rate of PAD. ALA can be further metabolized into LC n-3 PUFAs and lipid signaling molecules, which occurs in a complex biological pathway by enzymes that may be influenced by a combination of several factors including sex, genetics and background diet[45] that potentially could influence disease risk in a non-linear manner.

Previous studies have suggested that high intakes of the major n-6 PUFA linoleic acid and LC n-3 PUFAs may lower the conversion efficiency of ALA into LC n-3 PUFAs due to inhibition on shared enzymes. The median intake of LC n-3 PUFAs in this cohort was 0.7 g/d, which was higher than in cohort studies reporting inverse associations between ALA intake and CHD[15,16,18–20] and this may be of importance for our study findings because a large study has suggested that ALA may reduce CHD risk in particular when intake of LC n-3 is low[17]. However, further well-powered studies investigating the role of genetics and intake of LC n-3 and n-6 PUFAs on the association between ALA and the risk of atherosclerotic cardiovascular disease are warranted.

In conclusion, dietary intake of ALA was not consistently associated with the risk of incident PAD among Danish middle-aged men and women.

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Conflicts of interest: None

Authorship:

All authors contributed to the conceptualization of the present study. CSB conducted the statistical analyses, prepared the tables and figures, and wrote the first draft of the manuscript. CSB, ANL, SLC, MUJ, PCC, EBS and KO contributed to the planning of the statistical analyses, interpretation of the data and writing of the manuscript. SLC supervised the conduct of the statistical analyses. AT contributed to the interpretation of the data and writing of the manuscript. All authors have read and approved the final manuscript.

Figure text:

Figure 1. Flowchart of participants in the Diet, Cancer and Health cohort and incident cases of PAD identified during follow-up.

Figure 2. Intake of ALA and the risk of incident PAD. The multivariable analyses were conducted using Cox proportional hazard regression including adjustment for established PAD risk factors (model 1B) with the median intake of ALA as reference. The 20th, 40th, 60th and 80th percentiles of ALA intake are shown with dotted lines. The shaded grey area indicates the 95% CIs of hazard ratios of PAD (solid black line). The spline plot is shown for the 2.5-97.5 percentiles of ALA intake.

Table 1. Baseline characteristics

	Cohort (n = 55,248)	PAD cases (n = 950)
Sex, %		
Males	47.7	62.1
Females	52.3	37.9
Age at enrolment (years)*	56.1 (50.5; 64.7)	58.6 (50.7; 64.9)
Length of schooling (%)		
≤7 years	32.7	48.0
8-10 years	46.2	40.2
>10 years	21.1	11.8
Smoking (%)		
Never	35.4	4.8
Former	28.9	18.0
Current <15 g/d	13.0	19.0
Current 15-25 g/d	16.0	40.4
Current >25 g/d	6.8	17.7
Physical activity (%)		
Inactive	10.8	16.3
Moderately inactive	30.4	32.0
Moderately active	24.2	21.2
Active	34.7	30.5
Waist circumference (cm)*	89.0 (67.0; 115.0)	91.3 (68.8; 117.0)
Body mass index (kg/m^2)*	25.5 (19.6; 35.7)	25.5 (19.3; 34.7)
Alcohol intake (g/d)*	12.9 (0.2; 81.0)	16.5 (0.0; 104.7)
Co-morbidities (%)		
Hypercholesterolemia	7.4	13.8
Hypertension	16.1	27.8
Diabetes mellitus	2.0	10.7
Dietary factors		
Total energy intake (kJ)*	8895.2 (4937.2; 15142.9)	8749.6 (4730.1; 15537.0)
Intake of fiber*†	20.6 (12.3; 31.9)	19.3 (11.1; 29.9)
Glycemic load*	189.8 (98.8; 333.3)	182.7 (93.5; 335.9)
Saturated fatty acids*†	28.7 (16.4; 42.4)	31.4 (18.5; 44.2)
Monounsaturated fatty acids*†	28.1 (16.3; 43.3)	31.3 (18.3; 46.0)
Linoleic acid*†	11.0 (5.7; 20.1)	11.4 (6.2; 20.0)
LC n-3 PUFAs*†	0.7 (0.1; 1.7)	0.7 (0.2; 1.8)
Alpha-linolenic acid*†	1.8 (0.9; 3.3)	1.9 (1.0; 3.4)

*Median (2.5th; 97.5th percentile)

† Energy-adjusted intake

Table 2. Quintiles of energy-adjusted ALA intake and hazard ratios for peripheral artery disease

Quintiles of ALA intake	Cases (n)	Model 1A* HR (95% CI)	Model 1B † HR (95% CI)	Model 2‡ HR (95% CI)	Model 3§ HR (95% CI)
< 1.36 g/d	116	1 (reference)	1 (reference)	1 (reference)	1 (reference)
1.36-1.62 g/d	154	1.24 (0.98; 1.58)	1.08 (0.85; 1.38)	1.10 (0.86; 1.40)	0.98 (0.75; 1.27)
1.62-1.90 g/d	199	1.44 (1.14; 1.83)	1.14 (0.91; 1.45)	1.18 (0.93; 1.49)	0.99 (0.76; 1.30)
1.90-2.27 g/d	228	1.50 (1.18; 1.90)	1.10 (0.87; 1.40)	1.14 (0.90; 1.45)	0.92 (0.69; 1.23)
> 2.27 g/d	253	1.62 (1.27; 2.05)	1.05 (0.83; 1.34)	1.11 (0.87; 1.41)	0.88 (0.65; 1.20)

Abbreviations: ALA, alpha-linolenic acid; HR, hazard ratio

Statistical analyses were conducted using Cox proportional hazard regression. All models were adjusted for gender by allowing baseline hazards among men and women to differ.

* Model 1A included baseline age

† Model 1B included the variables of model 1A and the following risk factors for PAD: length of schooling, smoking, physical activity, waist circumference, body mass index and alcohol intake.

‡ Model 2 included the variables of model 1B and the following potential intermediate variables: self-reported history of hypercholesterolemia and/or use of lipid-lowering medication, hypertension and/or use of antihypertensive medication and diabetes mellitus and/or use of insulin.

§ Model 3 included the variables of model 1B and the following potential dietary risk factors: total energy intake, intake of fiber, glycemic load, and intake of saturated fatty acids, monounsaturated fatty acids, linoleic acid and LC n-3 PUFAs.

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