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BY
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AALBORG UNIVERSITY
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Christian Bork
July 2019

LIST OF PAPERS

This thesis is a summery based on the following five papers:

Paper I
Bork CS, Jakobsen MU, Lundbye-Christensen S, Tjønneland A, Schmidt EB & Overvad K. Dietary intake and adipose tissue content of alpha-linolenic acid and risk of myocardial infarction: a Danish cohort study. American Journal of Clinical Nutrition. 2016;104(1):41-48.

Paper II
Bork CS, Venø SK, Lundbye-Christensen S, Jakobsen MU, Tjønneland A, Schmidt EB & Overvad K. Dietary intake of alpha-linolenic acid is not appreciably associated with the risk of ischemic stroke among middle-aged Danish men and women. Journal of Nutrition. 2018;148(6):952-958.

Paper III
Bork CS, Venø SK, Lundbye-Christensen S, Jakobsen MU, Tjønneland A, Calder PC, Overvad K and Schmidt EB. Adipose tissue content of alpha-linolenic acid and the risk of ischemic stroke and ischemic stroke subtypes: a Danish case-cohort study. PLoS ONE. 2018;13(6):e0198927.

Paper IV
Bork CS, Lasota AN, Lundbye-Christensen S, Jakobsen MU, Tjønneland A, Calder PC, Schmidt EB and Overvad K. Intake of alpha-linolenic acid is not consistently associated with a lower risk of peripheral artery disease: results from a Danish cohort study. British Journal of Nutrition 2019 (Epub ahead of print)

Paper V
Bork CS, Lasota AN, Lundbye-Christensen S, Jakobsen MU, Tjønneland A, Overvad K and Schmidt EB. Adipose tissue content of alpha-linolenic acid and development of peripheral artery disease: a Danish case-cohort study. Submitted.

ABRREVIATIONS

ALA, Alpha-linolenic acid

ASCVD, Atherosclerotic cardiovascular disease

CHD, Coronary heart disease

DCH, Diet, Cancer and Health

DNPR, Danish National Patient Register

DHA, Docosahexaenoic acid

DPA, Docosapentaenoic acid

EPA, Eicosapentaenoic acid

FFQ, Food frequency questionnaire

HR, Hazard rate ratio

ICD, International Classification of Diseases

LA, Linoleic acid

MI, Myocardial infarction

PUFAs, Polyunsaturated fatty acids

Q, Quintile

TOAST, Trial of Org 10172 in Acute Stroke Treatment

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CHAPTER 1. INTRODUCTION

Atherosclerotic cardiovascular disease (ASCVD) including myocardial infarction (MI), ischemic stroke and peripheral artery disease (PAD) remain the leading cause of death globally (1,2). Despite major advances in the understanding of the underlying disease process of atherosclerosis and identification of several factors associated with disease risk, the burden of ASCVD remain high (2).

Nutrition represent an important area of research for primary prevention of ASCVD because biologically relevant dietary exposures in the human diet may have the potential to influence disease risk over decades.

N-3 polyunsaturated fatty acids (PUFAs) are organic compounds, which may affect a variety of biological processes of relevance to human health (3). The potential health benefits of n-3 PUFAs found in seafood has been subject to extensive research since the 1970s where Dyerberg and colleagues from Aalborg hypothesised that long-chain (LC) marine n-3 PUFAs might protect against atherosclerosis and thrombosis based on their expeditions to Greenland (4,5). Since then several epidemiological studies in other populations and some randomized clinical supplementation trials have supported the hypothesis that LC n-3 PUFAs may lower the risk of coronary heart disease (CHD) (6–9).

The potential health benefits of the plant-derived n-3 PUFA, alpha-linolenic acid (ALA), have been less studied and results in relation to ASCVD have been conflicting (10). However, apart from potential beneficial effects of ALA per se, ALA has also attracted public health relevance because it has been suggested as an alternative and a more sustainable source of LC n-3 PUFAs compared to LC n-3 PUFAs mainly derived from fatty fish and/or fish oil supplements (11,12). Also, ALA has been suggested to be an important mediator of a protective effect on CHD provided by the Mediterranean diet (13–15).

This thesis aimed to explore the associations between ALA and the risk of developing MI, ischemic stroke and PAD using complementary measures of exposure including estimated ALA intakes and adipose tissue content of ALA as a biomarker of its intake and metabolism. The included papers are based on data from the Danish Diet, Cancer and Health (DCH) cohort, which is a cohort of more than 57,000 middle-aged Danish men and women.

CHAPTER 2. BACKGROUND

2.1 ATHEROSCLEROSIS AND CARDIOVASCULAR DISEASE

Atherosclerosis is the main underlying disease process that may result in development of ASCVD. The atherosclerotic process begins in early life and may gradually progress with age under major influence by genetics, diet and lifestyle (16,17).

Progression of atherosclerosis and formation of atherosclerotic plaques are complex processes that occur in the arterial wall (18,19). In brief, the atherosclerotic process is believed to be initiated by endothelial dysfunction, retention of low-density lipoprotein (LDL) particles in the sub-endothelial layer and inflammation (18). The inflammatory response results in expression of adhesion molecules, chemokines and other pro-inflammatory cytokines, which leads to attraction of circulating leukocytes, especially monocytes, and mediates their migration and differentiation into macrophages in the arterial wall (18,19). The macrophages engulf the trapped and minimally modified and oxidized LDL particles and then becomes lipid rich foam cells. These lipid-laden foam cells trapped in the sub-endothelial layer continues internalization of atherogenic lipoproteins, which may be followed by apoptosis and development of a necrotic core within the atherosclerotic plaque (18,19).

Atherosclerotic plaque growth may over time result in narrowing of the arterial lumen, which gradually may limit the blood flow and hereby result in ischemia due to insufficient oxygen supply. Stable ASCVD may in the coronary arteries present as angina pectoris; or in the peripheral arteries in the legs as muscle discomfort or pain that typically is provoked by exercise and relieved by rest. However, atherosclerotic plaques may disrupt resulting in thrombus formation and sudden total or partial occlusion of the arterial lumen that may lead to acute critical ischemia in the affected arterial territory. In the cerebral arteries, acute ischemia may result in a transient ischemic attack or an ischemic stroke with rapid onset of focal neurologic dysfunction (20), whereas ischemia in the coronary arteries may present with various combinations of symptoms such as chest pain or upper extremity, mandibular or epigastric discomfort during exertion or at rest (21). In the peripheral arteries, acute ischemia mediated by atherothrombosis may present with symptoms as intermittent claudication or in later stages severe ischemic rest pain and gangrene (22).

The majority of cases of PAD and MIs are caused by atherosclerosis, whereas ischemic stroke is a more heterogeneous condition, which can be divided into subtypes according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification system based an assumed etiology (23). The TOAST classification system separates ischemic strokes into five groups including large artery atherosclerosis, small-vessel occlusion, cardio-embolism, stroke of other etiology and stroke of undetermined etiology (23). In the context of atherosclerosis, ischemic

strokes due to large artery atherosclerosis and ischemic strokes due to small vessel-occlusion are most relevant. Ischemic strokes due to large artery atherosclerosis is believed to be caused mainly by atherosclerosis within the larger arteries (23), whereas ischemic stroke due to small vessel-occlusion may occur as a result of atherosclerosis or lipohyalinosis affecting the smaller penetrating arteries (24).

A lowering of plasma LDL-cholesterol has convincingly been shown to reduce atherosclerosis and risk of ASCVD (25,26). Also, the role of inflammation has been considered a pivotal pathophysiological component of atherosclerosis for decades and is also believed to be important for the stability of atherosclerotic plaques and hence the risk of disruption resulting in acute events of ASCVD (27,28). Thus, targeting inflammation has been hypothesised as a way to reduce the risk of ASCVD (29). Interestingly, a recent clinical trial suggested targeting the pro-inflammatory cytokine interleukin-1β with an monoclonal antibody in subjects with a prior MI and elevated levels of C-reactive protein was associated with a lower rate of recurrent cardiovascular events compared with subjects receiving placebo (30).

2.2 POLYUNSATURATED FATTY ACIDS

PUFAs are fatty acids characterized by two or more double bindings in their carbon chain and are named according to the number, configuration and position of such double bonds (8). PUFAs has traditionally been divided into n-3 and n-6 PUFAs based on the position of the first double bond counted from the methyl end of the carbon chain (8). These PUFA families each consist of fatty acids with varying carbon chain length and degree of unsaturation, which are important for their pathophysiological and biological properties.

The shorter-chain n-3 PUFA ALA (18:3n-3) and the n-6 PUFA linoleic acid (LA, 18:2n-6) are the most abundant PUFAs in the typical Western diet. ALA is synthesized in plants and is found in high concentrations in walnuts and plant oils based on canola, soybean and flaxseed (12). However, ALA can also be found in other foods such as green leafy vegetables, whole-grain cereals, margarines, mayonnaises, dairy products and meat (31–33). LA is the most widely consumed n-6 PUFA found in many food sources, but the quantitively most important are plant oils and commercially available food products containing such oils, meat and eggs (34). The typical intake of PUFAs varies substantially by country, but most Western populations consume on average 0.5 to 2.3 g/d of ALA and 10-17 g/d of LA (12,34,35).

PUFAs may upon ingestion become available for energy production, incorporation into cells membranes and pools for storage (e.g. adipose tissue) or conversion into longer and more unsaturated fatty acids (12,36). ALA and LA are both considered essential fatty acids because humans lack the capacity to insert double bonds in the

n-6 and n-3 position necessary for production of these fatty acids endogenously. However, ALA and LA are both precursors for longer and more unsaturated fatty acids (37). ALA may thus be converted into the longer-chain n-3 PUFAs eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), whereas LA can be converted to arachidonic acid (20:4n-6) and fatty acids and beyond (Figure 1) (12,37).

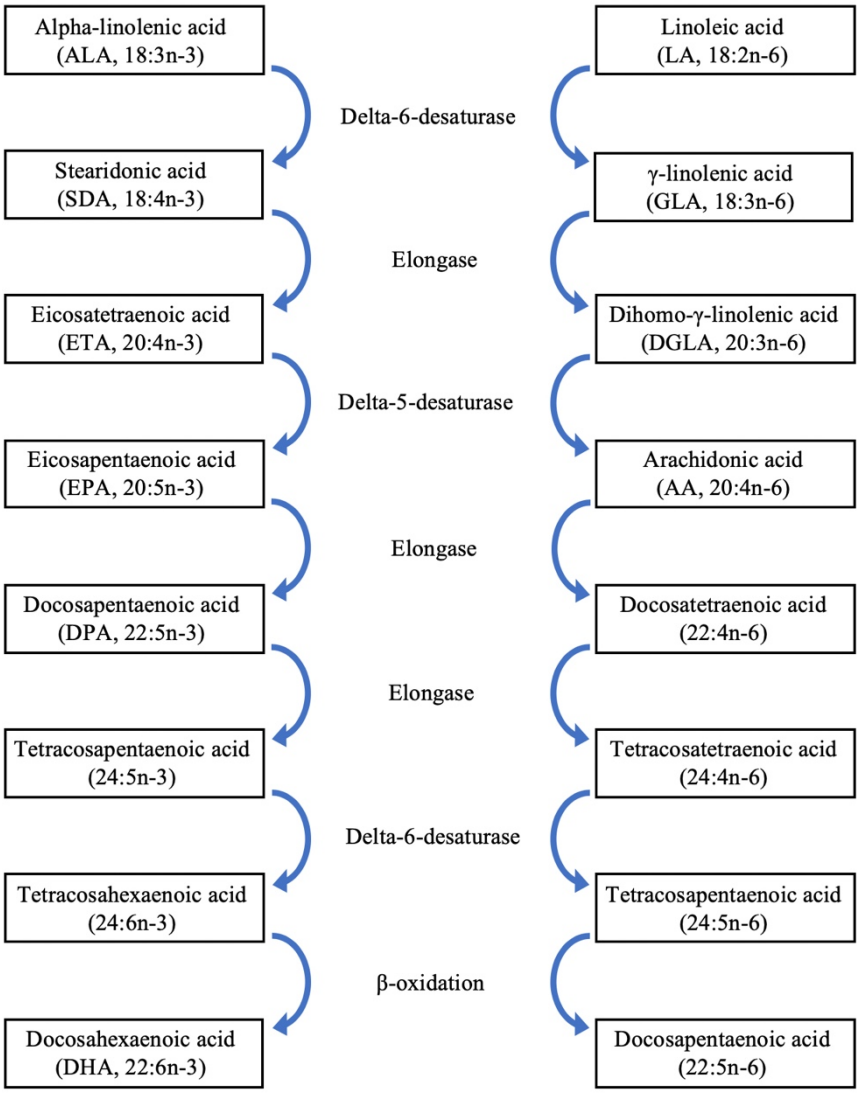


Figure 1. The pathway for synthesis of longer-chain PUFAs.

The conversion of ALA and LA into longer-chain fatty acids occurs in a complex pathway by shared enzymes, but no interconversions occurs between the two PUFA families (Figure 1). The conversion efficiency of ALA into LC n-3 PUFAs appears to be very limited in humans with reported conversions of ALA to EPA of less than 10% and to DHA of less than 1% (12,37). However, women and especially those of fertile age may be more efficient to convert ALA into LC n-3 PUFAs compared to men (38). Also, high intakes of LA or LC n-3 PUFAs may inhibit the metabolism of ALA due to competitive mechanisms (39–41). Although the conversion efficiency of ALA may be limited, the absolute contribution to the endogenous exposure of predominantly EPA derived from ALA may be of significance due to a markedly higher intake of ALA than LC n-3 PUFAs in most populations.

PUFAs are biologically active compounds that may affect a myriad of molecular pathways such as alteration of physical and chemical properties of cellular membranes and direct effects on ion channels, gene expression and biosynthesis of lipid signalling molecules and other mediators (8,42). Important lipid signalling molecules not least eicosanoids including prostaglandins, thromboxanes and leukotrienes, which are involved in regulation of several biological functions important for vascular function, thrombosis and inflammatory processes (3,42). Both PUFA families may produce inflammatory signaling molecules, but the n-3 PUFAs have been ascribed anti-inflammatory effects, and the lipid signaling molecules derived from the n-3 PUFAs are considered less proinflammatory than those derived from n-6 PUFAs (3).

CHAPTER 3. AIMS AND HYPOTHESES

The objective of this thesis was to investigate the associations between the energy-adjusted intake of ALA and adipose tissue content of ALA and the rate of MI, ischemic stroke and PAD.

We hypothesized that estimates of ALA exposure were inversely associated with the rate of MI, ischemic stroke and PAD.

CHAPTER 4. METHODS

The methods used have been described in detail in the five papers included in this thesis and will only be described in brief below.

4.1 STUDY POPULATION

The studies included in this thesis were based on data from the DCH cohort. The cohort was established to investigate diet and lifestyle in relation to development of cancer and other chronic diseases (43). The study participants were recruited from in and around the two largest cities in Denmark, Copenhagen and Aarhus, by letter invitations. The criteria for invitation were as follows (43):

1. Age between 50 and 64 years
2. Born in Denmark
3. No previous diagnosis of cancer registered in the Danish Cancer Registry

Potentially eligible study participants were retrieved using the Danish Civil Registration System and the enrolment began in December 1993 and ended in May 1997 (43). A total of 160,725 participants including 80,996 men and 79,729 women were invited to participate in the study, which corresponded to nearly 20% of the Danish population aged 50 to 64 years at the time of invitation. A total of 57,053 participants (27,178 men and 29,875 women) accepted the invitation (43). A randomly drawn sample of 3,500 participants was drawn from the cohort to assess the exposure distribution for biomarkers studies of fatty acids.

Processing delay in the registration of diagnoses of cancer in the Danish Cancer Registry resulted in invitation of some participants that had developed cancer. All participants registered with a diagnosis of cancer before enrolment were excluded from the analyses included in this thesis. Also, participants with a previous diagnosis of the relevant outcomes of interest were excluded in order to study the associations between ALA exposure in relation to development of ASCVD. In analyses of PAD, we additionally excluded participants with a previous diagnosis of chronic kidney insufficiency because these patients might develop atypical or more aggressive forms of PAD to whom the main results might not be generalised to. Finally, participants with missing information on exposures and/or covariates were excluded assuming that these were missing at random.

All participants gave written informed consent at inclusion, and the study was approved by the relevant Ethic Committees and the Data Protection Agency.

4.2 ASSESSMENT OF DIETARY INTAKE OF ALA

All participants received a 192-item food frequency questionnaire (FFQ) by mail and was asked to report their average intake the past year of foods and beverages within 12 possible response categories ranging from never to 8 times or more per day (43). The FFQs were optically scanned and checked for missing values and uncertainties and clarified by technicians at a physical examination performed at two study centres (43). The daily intake of ALA and other nutrients were estimated for each participant using the software program FoodCalc based on standardized recipes and portion sizes. The FFQ was developed during the planning of the DCH cohort (44) and has previously been validated against two times 7-day weighted diet records and found suitable to categorise subjects according to their intake of energy and PUFAs (45).

4.3 ASESSMENT OF ADIPOSE TISSUE CONTENT OF ALA

All participants were asked to have a subcutaneous adipose tissue biopsy taken from the buttocks during the physical examination. The adipose tissue biopsies were collected using a Luer-lock system consisting of a needle, a venoject multi-sample Luer adapter and an evacuated blood tube according to the method of Beynen and Katan (46). Samples were stored in liquid nitrogen vapour until analysis. The fatty acid composition of adipose tissue was determined by gas chromatography and expressed as area percentage of total fatty acids. The method used for fatty acid analysis has previously been described in detail in the individual papers included in the thesis and elsewhere (47).

4.4 ASSESSMENT OF COVARIATES

All participants were asked to fill in a questionnaire during the physical examination on social and lifestyle factors such as length of schooling, smoking habits and physical activity. Also information on hypercholesterolemia, hypertension or diabetes mellitus and relevant medications used to treat these conditions were collected (43). The questionnaires were checked for reading errors and missing information by technicians who also performed anthropometric measurements such as height, weight and waist circumference of the participants (43). Information on alcohol intake and dietary covariates was derived from the FFQ. Information of a history of atrial fibrillation/flutter and history of chronic kidney insufficiency prior to baseline was not collected during the recruitment process. Instead, history on atrial fibrillation/flutter (International Classification of Diseases 8th revision (ICD-8): 42793, 42794 & ICD-10: DI48) and chronic kidney insufficiency (ICD-8: 79299 & ICD-10: DN18, DN181, DN182, DN183, DN184; DN185, DN188 and DN189)

was obtained by record linkage with the nationwide Danish National Patient Register (DNPR).

4.5 OUTCOME ASSESSMENT

The case ascertainment in the presented papers was based on record linkage between the cohort participants and discharge diagnoses registered in the DNPR. This administrative register was established in 1977 and has gradually been expanded with more information over the years (48,49). Initially, the DNPR covered all inpatient contacts to Danish hospitals, but from 1995 and onwards all outpatient activities and emergency room contacts were included as well. Primary and secondary discharge diagnoses were classified in the DNPR according to the ICD-8 until the end of 1993, and according to the ICD-10 from then (49).

Myocardial infarction

Participants registered with a primary or secondary discharge diagnosis of MI (ICD-8: 410-410.99, or ICD-10: DI210–DI219) or cardiac arrest (ICD-8: 427.27 or ICD-10: DI460–DI469) registered in the DNPR or the Danish Causes of Death Registry (50) until July 2013 were identified (51). All potential cases identified from baseline through 2003 were validated by review of medical records. All cases of cardiac arrest were included as cases if the arrest was considered to be of cardiac origin after validation. From January 2004 and onwards all participants registered with a diagnosis of MI from a hospital ward was accepted as cases, because the positive predictive value of a diagnosis of MI was shown to be above 92% after validation of more than 800 of the first registered MI cases (51). All other diagnoses were validated by examining a complete list of relevant diagnoses and procedure codes recorded in the DNPR for each individual participant. The cohort was followed from baseline until the first registration of MI, death, emigration or end of follow-up in July 2013.

Ischemic stroke

Participants registered with a primary or secondary discharge diagnosis of stroke (ICD-8: 430, 431, 433, 434, 436.01, or 436.90, or ICD-10: DI60, DI61, DI63, or DI64) in the DNPR until November 2009 were identified (52). All potential stroke cases were validated by review of medical records and subsequently subtyped into ischemic stroke subtypes according to the TOAST classification (23) based on clinical findings, brain imaging, imaging of extracranial arteries, laboratory tests, electrocardiograms, and echocardiography (52). The cohort was followed from baseline until first registration of stroke, death, emigration or end of follow-up in November 2009.

PAD

Participants registered with a primary or secondary discharge diagnosis of PAD (ICD-8: 44390, 44500, 44509, 44590, 44599, 44020 or 44030, or ICD-10: DI702, DI702A and DI739A-C) until December 2009 were identified. Subsequently, all potential cases of PAD were validated by review of medical records (53). The cohort was followed from baseline until first registration of PAD, death, emigration or end of follow-up in December 2009.

4.6 METHODOLOGICAL CONSIDERATIONS

Study designs

We used a traditional follow-up strategy to investigate the associations between ALA intake and the rate of MI, ischemic stroke and PAD, while a case-cohort design was used to investigate associations between adipose tissue content of ALA and the rate of MI, ischemic stroke and PAD (Figure 2). In a case-cohort design, the study is nested within a cohort followed over time, but the exposure information is only required from cases and from a randomly drawn sample of the cohort. During statistical analysis, the random sample representing the exposure and covariates distribution in the total cohort can be weighted to adjust for the overrepresentation of cases in the case-cohort dataset. In the DCH cohort a randomly drawn sub-cohort of 3,500 participants was used to represent the adipose tissue content of ALA and covariate distribution within the whole cohort. The major advantage of the case-cohort design is that the sub-cohort can be used as a comparison group for studying different diseases without identifying a new set of controls for each disease as required in a nested case-control study. The use of a case-cohort design in our studies limited the costs for the number of expensive fatty acid analyses of adipose tissue samples significantly.

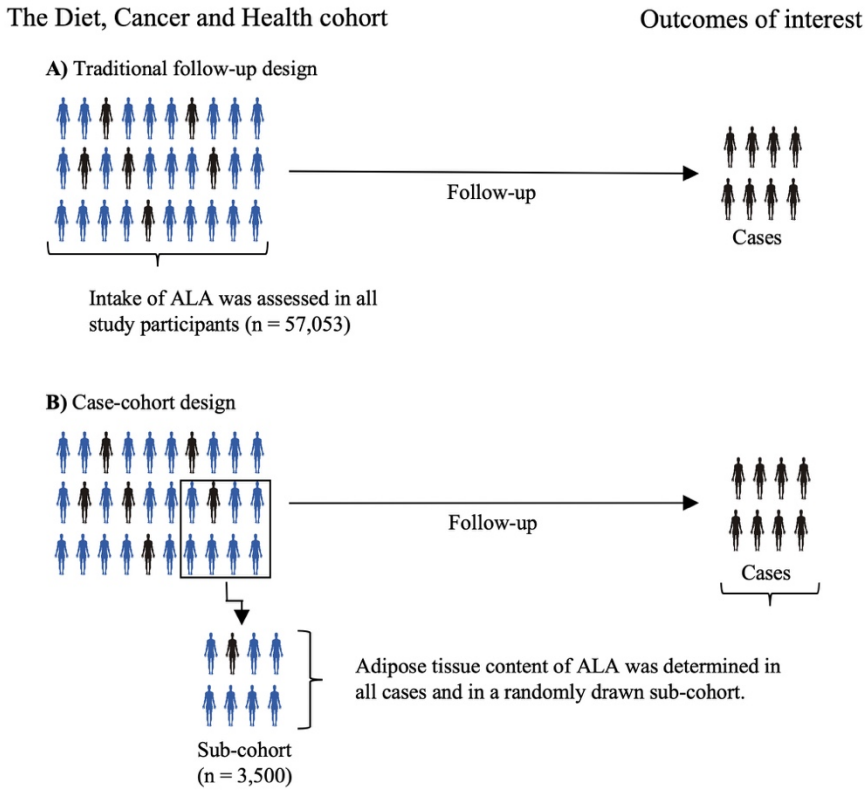


Figure 2. Illustration of the principle of a traditional follow-up design and a case-cohort design.

Exposures of interest

In analyses of dietary intakes of ALA, we decided to energy-adjust intake of ALA using the residual method (54). Total energy intake is largely determined by body size, physical activity, and metabolic efficiency and is typically relative constant unless changes in weight or physical activity occur (55). Thus, intake of ALA in individuals is likely to be altered mainly by changing the composition of the diet rather than the amount of food. In addition, studies have shown that total energy intake may be inversely associated with the risk of CHD possibly mediated by health benefits of physical activity and adjustment for total energy intake may be important for reducing confounding (55).

Adipose tissue content of ALA was expressed as area percentage of the total of fatty acids in adipose tissue (relative fatty acid concentrations are often used in biomarker studies of fatty acids).

Risk factors

A large number of risk factors have been documented or proposed for MI and ischemic stroke, while risk factors for PAD have been less studied. Identification of risk factors in the papers included in this thesis was based on review of the literature and potential risk factors were pre-specified prior to data analysis. An overview of the covariate adjustments used is presented in Table 1. Shared and established risk factors for MI, ischemic stroke and PAD include age, educational level, physical activity, alcohol consumption, hypercholesterolemia, hypertension and diabetes mellitus. Although, MI, ischemic stroke and PAD may share pathophysiological similarities, differences in associated risk factors and their importance for disease risk may differ among these outcomes. Studies have suggested that the risk of MI may differ by sex (56,57), while the association between sexes and ischemic stroke and PAD is less clear. Therefore, we decided to conduct analyses of MI separately among men and women (sex-specific), whereas sex-stratified analyses were conducted for analyses of ischemic stroke and PAD by allowing for different baseline hazards among men and women. In addition, sex-specific analyses would not have allowed for investigation of associations with ischemic stroke subtypes and a detailed control for confounding in analyses of ischemic stroke subtypes and PAD due to a relatively low number of female cases. Menopausal status and use of hormone replacement therapy have been suggested as risk factors for MI (58,59), whereas their role in ischemic stroke and PAD is less clear. With respect to atrial fibrillation/flutter this is an important risk factor for ischemic stroke, but not for MI and PAD. Age is probably the most important risk factor for MI, ischemic stroke and PAD and was therefore taken into account in all analyses. In addition, we decided to include baseline age as a covariate in analyses on ischemic stroke and PAD to ensure comparison of participants for whom exposure information was of comparable age.

During review of the literature, we identified several potential dietary risk factors that might influence CHD risk including total energy intake, glycemic load and dietary intakes of fibre, monounsaturated fatty acids, saturated fatty acids, LA and LC n-3 PUFAs.

Statistical models

We defined four different models as presented in Table 1. Model 1A was controlled for age and sex, while model 1B included additional adjustment for established risk factors. The minimally adjusted model 1A was presented in order to evaluate the importance of potential confounding by comparing with the point estimates of model 1B as large differences might indicate confounding. However, no conclusions should be drawn from the minimally adjusted model 1A due to the risk of confounding.

In model 2, we included the covariates in model 1B and co-morbidities, which are considered important risk factors for the outcomes of interest. However, the rather broad range of proposed mechanisms for the effects of ALA imply that these co-morbidities may be considered potential intermediate steps in the causal pathway between ALA and the risk of the outcomes of interest.

The interpretation of model 2 is complicated because adjustment for these co-morbidities in part might eliminate the pathway through these mediators resulting in attenuation of observed associations and/or introduce collider stratification bias. However, these potential mediators are presumably only affected by ALA to a limited extent and not taking the presented comorbidities into account might result in residual confounding.

In supplemental analyses of dietary intake of ALA, we adjusted for established risk factors and potential dietary risk factors including total energy intake, glycemic load and dietary intake of fibre, monounsaturated fatty acids, saturated fatty acids, LA and LC n-3 PUFAs (model 3) in order to address potential confounding from the diet. However, measures of association obtained from analyses including adjustment for diet are difficult to interpret because restrictions into the underlying dietary pattern are introduced and these differences may not be reasonable within ordinary dietary patterns limiting the public health relevance. Therefore, given the interpretational complexities of model 2 and 3, we consider model 1B the most appropriate for interpretation.

Table 1. Overview of the covariate adjustments according to outcomes of interest.

	MI	Ischemic stroke	PAD
	sex-specific	sex-stratified	sex-stratified
Model 1A			
Baseline age (years, spline)		● 3kn	● 5kn
Model 1B			
Smoking (never; former; current (1-4, 15-24, >24 g/d))	●	●	●
Physical activity (inactive, moderately inactive, moderately active, active)	●	●	●
Length of schooling (≤7yrs; 8-10 years; >10yrs)	●	●	●
Waist circumference (cm, spline)	● 5kn		● 5kn
Body mass index (kg/m^2, spline)	● 5kn		● 5kn
Waist circumference adjusted for BMI (cm, spline)		● 3kn	
Menopausal status (pre; post; unknown)[1]	●		
Hormone replacement therapy (yes; no; unknown)[1]	●		
Alcohol intake (g/d, spline)	● 5kn	● 3kn	● 5kn
Model 2			
History of hypercholesterolemia (yes; no; unknown)	●	●	●
History of hypertension (yes; no; unknown)	●	●	●
History of diabetes mellitus (yes; no; unknown)	●	●	●
History of atrial fibrillation (yes; no; unknown)		●	
Model 3 (Supplemental analyses)[2]			
Total energy intake (kJ/day)	● 5kn	● 3kn	● 5kn
Glycemic load	● 5kn	● 3kn	● 5kn
Fibre (g/day)	● 5kn	● 3kn	● 5kn
Monounsaturated fatty acids (g/day)	● 5kn	● 3kn	● 5kn
Saturated fatty acids (g/day)	● 5kn	● 3kn	● 5kn
Linoleic acid (g/day)	● 5kn	● 3kn	● 5kn
LC n-3 PUFAs (g/day)	● 5kn	● 3kn	● 5kn

Abbreviations: Kn, knots (3 or 5) in a restricted cubic spline; BMI, Body mass index

[1] Women only

[2] Dietary analyses of ALA only

4.7 STATISTICAL ANALYSES

We used time-to-event analysis to investigate associations between dietary intake and adipose tissue content of ALA and the risk of MI, ischemic stroke or PAD. Hazard rate ratios (HRs) with 95% CIs, calculated using Cox proportional hazard regression, were used as measures of association. We used age as underlying time scale in all analyses.

We decided to assess the shape of the associations by analysing estimates of ALA exposure as a continuous variable by the use of restricted cubic splines. Categorical analyses using ALA divided into quintiles of exposure using the lowest quintile as

reference were also undertaken, but the continuous analyses were considered the primary analyses. We decided to express ALA exposure using restricted cubic splines with 3 knots placed at the 10th, 50th and 90th percentile as described by Harrell (60). The median ALA exposure was used as reference, and the spline functions were formally tested against a horizontal line using Wald's test. In sensitivity analyses, we investigated the robustness of the models by modifying the location of the knots and by increasing the number of knots.

The associations between adipose tissue content of ALA and the outcomes of interest were investigated using weighted Cox proportional hazard regression. Several different weighting schemes has been suggested, and we used the weighting scheme described by Kalbfleisch and Lawless (61) because this approach has previously been reported to perform well in a study that compared the most commonly used case-cohort estimators (62). Thus, all cases were assigned a weight equal to one, whereas all non-cases in the sub-cohort were assigned a weight calculated as the ratio between the number of non-cases in the cohort divided by the number of non-cases in the sub-cohort. Individual weights were calculated for each outcome of interest.

We entered the covariates into four different models as shown in Table 1. In analyses of MI and PAD, we included all continuous risk factors as restricted cubic splines using 5 knots to ensure the best possible adjustment for these covariates. However, in analyses of ischemic strokes we limited the complexity of the statistical models due to the relatively low number of cases of the most uncommon ischemic stroke subtypes by reducing the number of knots for continuous covariates to three and by including waist circumference adjusted for BMI instead of including waist circumference and BMI separately into the models.

The proportional hazard assumption was evaluated by plotting the scaled Schoenfeld residuals against age at event assessing potential violations visually using fractional polynomial regression for each covariate. An approximate horizontal line indicated constant hazard ratios over time and fulfilment of the proportional hazard assumption.

All statistical analyses were conducted using Stata statistical software and a p-value below 0.05 was considered statistically significant. Measures of association that did not reach conventional statistically significance was described as indications of associations when considered appropriate bearing in mind that statistically non-significant associations should not be interpreted as absence of associations (63).

The background dietary pattern was described using radar plots for a selected number of foods and beverages . Intake of foods was energy-adjusted using the residual method (54).

CHAPTER 5. STUDIES

5.1 OVERVIEW

We aimed to investigate the associations between the energy-adjusted dietary intake and adipose tissue content of ALA and the rate of incident ASCVD. We hypothesized that dietary intake and adipose tissue content of ALA were inversely associated with the rate of incident MI, ischemic stroke and PAD. These results of our studies have been reported in five papers:

Paper I: *Dietary intake and adipose tissue content of alpha-linolenic acid and risk of myocardial infarction: a Danish cohort study*.

Paper II: *Dietary intake of alpha-linolenic acid is not appreciably associated with the risk of ischemic stroke among middle-aged Danish men and women*.

Paper III: *Adipose tissue content of alpha-linolenic acid and the risk of ischemic stroke and ischemic stroke subtypes: a Danish case-cohort study*.

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

Paper V: *Adipose tissue content of alpha-linolenic acid and development of peripheral artery disease: a Danish case-cohort study***.**

In the following, the papers outlined above will be summarised according to the outcomes of interest including MI (paper 1), ischemic stroke (papers 2 and 3) and PAD (papers 4 and 5).

5.2 ALA AND MI

Aim

To investigate the associations between dietary intake and adipose tissue content of ALA and the rate of MI.

Key methods

Incident cases of MI were identified through the DNPR and the Danish Causes of Death Registry. The association between ALA intake expressed as energy-adjusted intake in g/d and MI was investigated using a follow-up design. The association between adipose tissue content of ALA and MI was investigated using a case-cohort design in which the adipose tissue content of ALA expressed as a percentage of total fatty acids in all cases and in a randomly drawn sample from the cohort (sub-cohort). Analyses were conducted separately among men and women and HRs obtained from Cox proportional hazard regression analyses were used as measures of association.

Main results

During 17 years of follow-up, 2,124 men and 854 women developed MI for whom adipose tissue biopsies were available in 1,994 men and 770 women (Figure 3 and Table 2).

In multivariable analyses with ALA intake modelled as a spline adjusted for traditional risk factors for MI (model 1B), an indication of a weak and statistically non-significant positive association was observed between ALA intake and the rate of MI in men, whereas an indication of a weak statistically non-significant U-shaped association was found in women. Analyses of the associations between quintiles of ALA intake and the rate of MI are summarized in Figure 4. Supplemental analyses including additional adjustment for dietary factors are presented in Appendix Figure 1.

Multivariable analyses with ALA content in adipose tissue modelled as a spline adjusted for established risk factors for MI (model 1B) indicated a statistically non-significant positive association between the content of ALA in adipose tissue and the rate of MI in men, whereas an indication of a weak statistically non-significant U-shaped association was observed in women. Analyses of the associations between adipose tissue content of ALA in quintiles and the rate of MI in men and women are given in Figure 4.

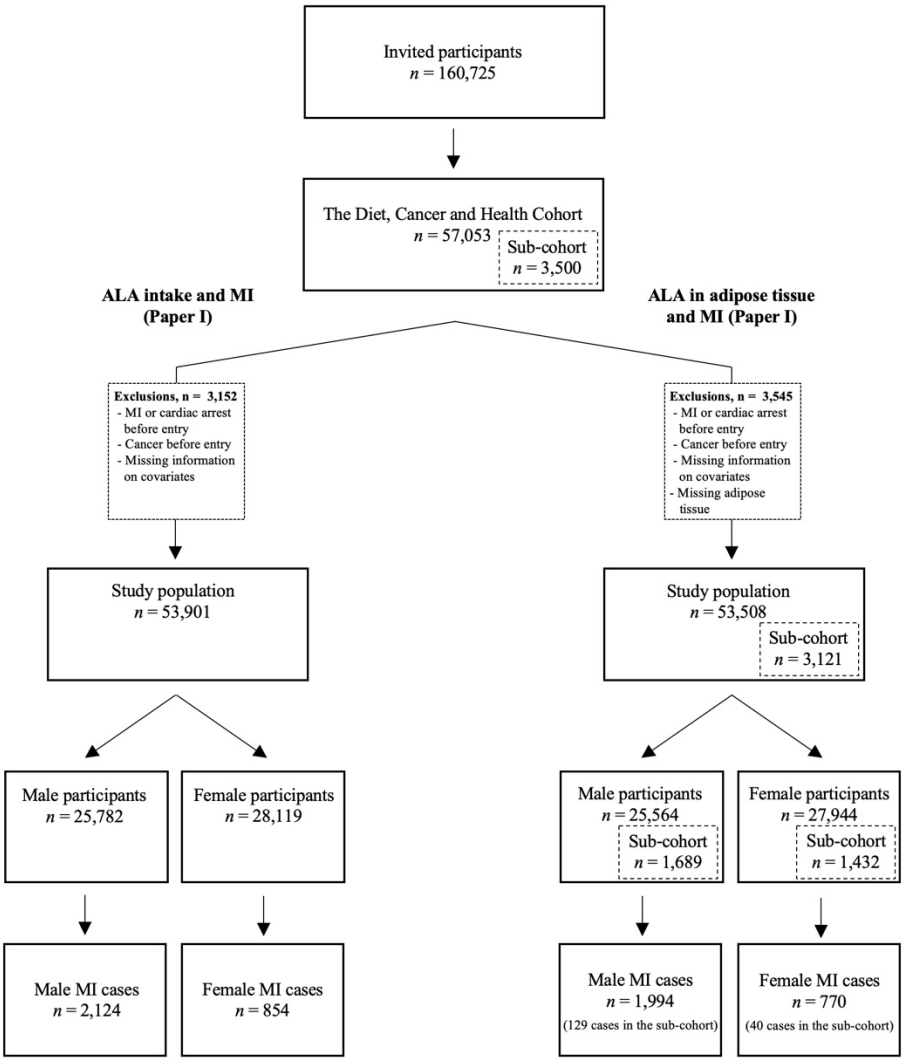


Figure 3. Flowchart for the DCH cohort and incident MI cases identified among men and women with complete information on exposures and other covariates of interest.

Table 2. Baseline characteristics of the cohort, sub-cohort and participants that developed MI during follow-up.

	ALA intake and MI				ALA in adipose tissue and MI			
	Men		Women		Men		Women	
	Cohort (n = 25,782)	Cases (n = 2,124)	Cohort (n = 28,119)	Cases (n = 854)	Sub-cohort (n = 1,689)	Cases (n = 1,994)	Sub-cohort (n = 1,432)	Cases (n = 770)
Age at enrolment (years)[1]	55.9	57.6	56.2	59.2	56.3	57.6	56.2	59.2
Postmenopausal (%)	-	-	58.3	68.5	-	-	58.4	68.7
Length of schooling (%)								
≤7 years	34.1	43.1	30.9	43.9	34.1	43.3	30.9	43.4
8-10 years	41.7	37.3	50.4	46.5	40.9	37.3	50.0	47.1
>10 years	24.1	19.6	18.7	9.6	25.0	19.4	19.1	9.5
Smoking (%)								
Never	26.2	18.2	43.9	26.8	26.8	18.5	44.6	27.9
Former	34.4	29.6	23.5	19.3	35.1	30.2	21.9	19.7
Current <15 g/d	10.6	12.3	15.2	22.4	10.9	12.5	16.1	21.9
Current 15-25 g/d	17.4	23.9	14.8	26.6	16.5	23.2	15.0	25.3
Current >25 g/d	11.5	16.0	2.6	4.9	10.6	15.7	2.4	5.1
Physical activity (%)								
Inactive	11.2	14.3	10.3	16.0	11.2	14.2	10.9	16.1
Moderately inactive	28.7	29.4	32.1	36.1	28.3	29.3	33.0	36.9
Moderately active	23.6	22.3	24.8	22.1	24.2	22.2	23.0	21.9
Active	36.5	34.0	32.9	25.8	36.3	34.2	33.2	25.1
Waist circumference (cm)[1]	95.0	97	80	84	95	97	80	84
Body mass index (kg/m^2)[1]	26.1	26.9	24.8	25.8	26.4	27.0	24.7	26.0
Alcohol intake (g/d)[1]	19.4	18.2	9.5	6.6	19.7	18.1	9.5	6.6
Hormone replacement therapy	-	-	30.8	30.1	-	-	30.4	29.7
Co-morbidities (%)								
Hypercholesterolemia	7.7	11.8	6.0	12.5	8.4	12.1	6.1	12.7
Hypertension	14.5	22.0	17.2	30.9	14.8	22.4	17.0	31.6
Diabetes mellitus	2.6	5.3	1.4	4.1	2.7	5.1	1.3	4.0

[1] Median

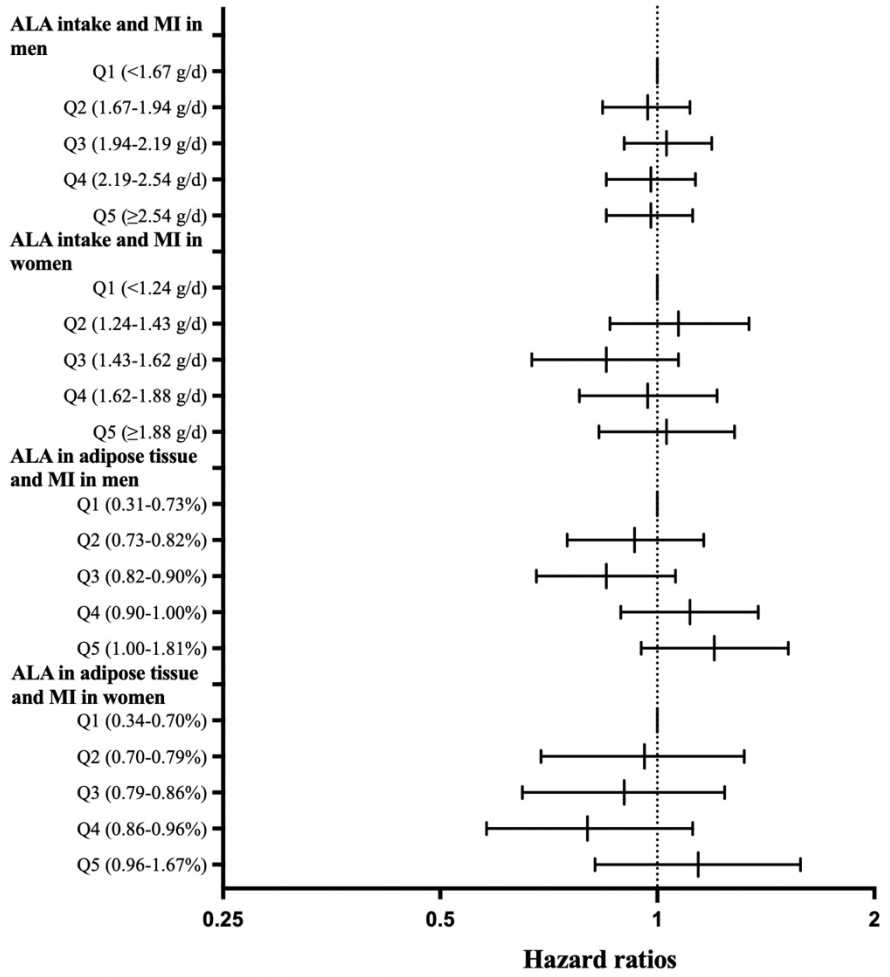


Figure 4. Forest plot of the associations between quintiles of energy-adjusted ALA intake and the rate of MI in analyses including adjustment for established risk factors (model 1B).

Conclusion

ALA intake was not appreciably associated with the rate of MI. An indication of a weak positive association between adipose tissue content of ALA was observed in men and a weak U-shaped association in women was observed, but these associations were not statistically significant.

5.3 ALA AND ISCHEMIC STROKE

Aim

To investigate the association between dietary intake and adipose tissue content of ALA and the rate of ischemic stroke and ischemic stroke subtypes.

Key methods

Incident cases of ischemic stroke were identified through the DNPR, validated and classified into subtypes according to the TOAST criteria. ALA intake was expressed as energy-adjusted intake in g/d and analysed using a follow-up design, whereas adipose tissue content of ALA was analysed using a case-cohort design. HRs obtained from Cox proportional hazard regression analysis were used to describe the associations between intake and adipose tissue content of ALA and the rate of ischemic stroke and its subtypes allowing for different baseline hazards among sexes.

Main results

During a median of 13.5 years of follow-up, 1,859 ischemic strokes occurred with content of ALA in adipose tissue available in 1,735 cases (Figure 5 and Table 3).

Multivariable analyses with ALA modelled as a spline adjusted for traditional risk factors for ischemic stroke (model 1B) indicated a weak statistically non-significant positive association between ALA intake and the rate of total ischemic stroke. In analyses of ischemic stroke subtypes, indications of a weak statistically non-significant inverse associations were observed between ALA intake and ischemic stroke due to large artery atherosclerosis (316 cases), whereas an indication of a weak statistically non-significant positive association was seen between ALA intake and ischemic stroke due to small-vessel occlusions (835 cases). Analyses of the associations with ALA intake in quintiles are presented in Figure 6. Analyses including additional adjustment for dietary factors are presented in Appendix Figure 1.

Multivariable analyses with ALA modelled as a spline adjusted for ischemic stroke risk factors (model 1B) indicated a statistically non-significant U-shaped association between ALA content in adipose tissue and the rate of total ischemic stroke. In analyses of ischemic stroke subtypes, a statistically significant U-shaped association was observed between adipose tissue content of ALA and the rate of ischemic stroke due to large artery atherosclerosis (297 cases), whereas no appreciable association was found between ALA content in adipose tissue and the rate of ischemic stroke due to small-vessel occlusions (772 cases). Analyses of the associations with ALA content in adipose tissue in quintiles are presented in Figure 6.

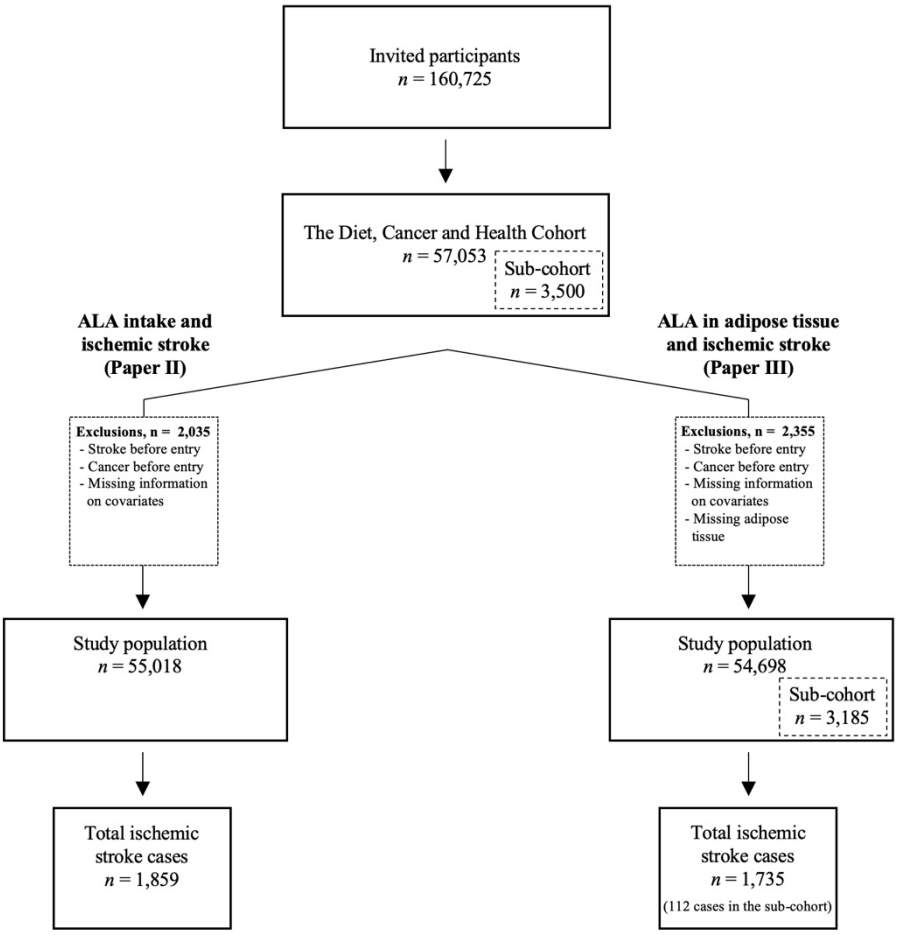


Figure 5. Flowchart for the DCH cohort and incident total ischemic stroke cases identified participants with complete information on exposures and other covariates of interest.

Table 3. Baseline characteristics of the cohort, sub-cohort and participants that developed ischemic stroke during follow-up.

	ALA intake and ischemic stroke		**ALA in adipose tissue and ischemic stroke**	
	Cohort (n = 55,018)	Cases (n = 1,859)	Sub-cohort (n = 3,185)	Cases (n = 1,735)
Sex (%)				
Men	47.6	61.7	54.1	61.8
Women	52.4	38.4	45.9	38.2
Age at enrolment (years)[1]	56.1	58.8	56.3	58.8
Length of schooling (%)				
≤7 years	32.7	40.4	32.7	40.7
8-10 years	46.1	42.7	45.0	42.7
>10 years	21.1	16.9	22.3	16.6
Smoking (%)				
Never	35.4	24.2	34.8	24.6
Former	28.8	25.4	29.3	25.8
Current <15 g/d	13.0	15.5	13.5	15.5
Current 15-25 g/d	16.1	24.2	15.7	23.8
Current >25 g/d	6.8	10.8	6.8	10.3
Physical activity (%)				
Inactive	10.7	14.9	11.0	14.7
Moderately inactive	30.3	30.3	30.4	30.2
Moderately active	24.2	21.8	23.7	21.4
Active	34.8	33.0	35.0	33.7
Waist circumference (cm)[1]	88.9	93.0	91.1	93.6
Alcohol intake (g/d)[1]	13.0	14.6	13.9	14.5
Co-morbidities (%)				
Hypercholesterolemia	7.3	10.4	7.8	10.7
Hypertension	16.0	28.0	15.6	28.4
Diabetes mellitus	2.0	4.5	2.0	4.2
Atrial fibrillation	0.7	1.5	0.9	1.4

[1] Median

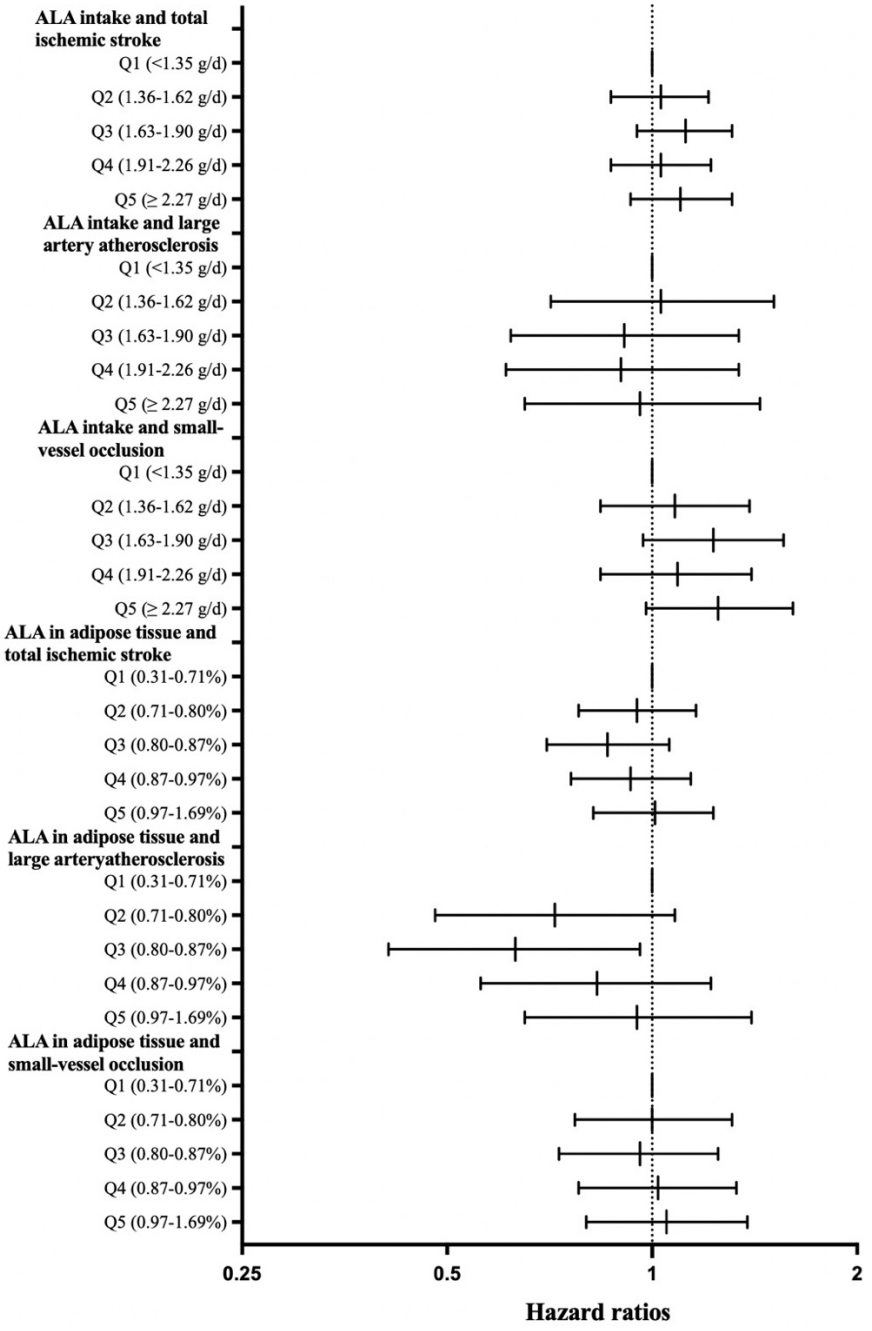


Figure 6. Forest plot of the associations between quintiles of ALA and the rate of ischemic stroke in analyses including adjustment for established risk factors (model 1B).

Conclusion

ALA intake was not consistently nor appreciably associated with the rate of total ischemic stroke or ischemic stroke subtypes of atherosclerotic origin. Adipose tissue content of ALA may be U-shapedly associated with the rate of total ischemic stroke and ischemic stroke due to large artery atherosclerosis, while no appreciable association was observed for ischemic strokes due to small-vessel occlusions.

5.4 ALA AND PAD

Aim

To investigate the associations between dietary intake and adipose tissue content of ALA and the rate of PAD.

Key methods

Incident cases of PAD were identified by linkage with the DNPR. Subsequently, all cases were validated. The energy-adjusted intake of ALA in g/d was calculated for each participant and an association with PAD was investigated using a follow-up design, while the association between adipose tissue content of ALA was determined in all cases and in the sub-cohort and analysed by a case-cohort design. We used HRs obtained from Cox proportional hazard regression to investigate the associations between intake and adipose tissue content of ALA and the rate of PAD allowing for different baseline hazards among sexes.

Main results

During a median of 13.5 years of follow-up, 950 cohort participants developed PAD with adipose tissue content of ALA being available in 863 cases (Figure 7 and Table 4).

In multivariable analyses with ALA modelled as a spline adjusted for traditional PAD risk factors (model 1B), an indication of a weak statistically non-significant inverse U-shaped association was observed between ALA intake and the rate of PAD. Analyses of the associations between ALA intake in quintiles and the rate of PAD are shown in Figure 8. Analyses including additional adjustments for dietary factors are presented in Appendix Figure 1.

Multivariable analyses with ALA modelled as a spline adjusted for traditional PAD risk factors (model 1B) indicated a statistically non-significant U-shaped association between ALA content in adipose tissue and the rate of PAD. Categorical analyses of associations between ALA intake in quintiles and the rate of PAD are shown in Figure 8.

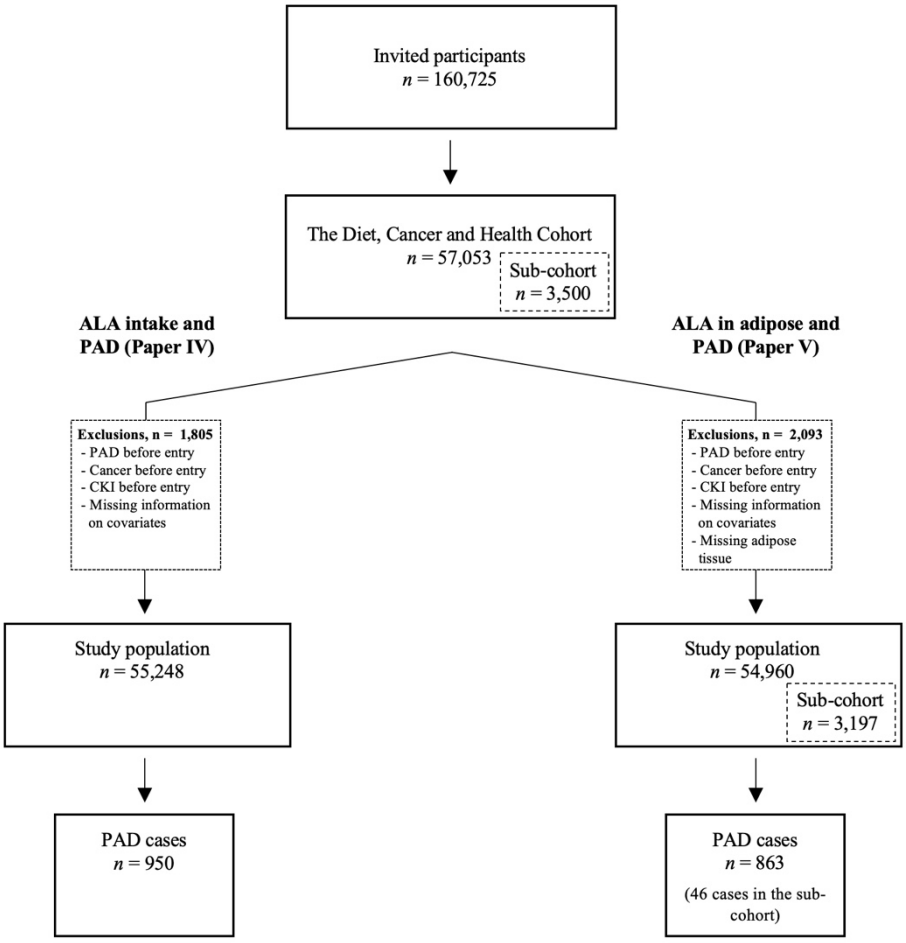


Figure 7. Flowchart for the DCH cohort and incident PAD cases identified among participants with complete information on exposures and other covariates of interest.

Table 4. Baseline characteristics of the cohort, sub-cohort and participants that developed PAD during follow-up.

	ALA intake and ischemic stroke		**ALA in adipose tissue and ischemic stroke**	
	Cohort (n =55,248)	Cases (n = 950)	Sub-cohort (n = 3,197)	Cases (n = 863)
Sex (%)				
Men	47.7	62.1	54.1	61.7
Women	52.3	37.9	46.0	38.4
Age at enrolment (years)[1]	56.1	58.6	56.3	58.5
Length of schooling (%)				
≤7 years	32.7	48.0	32.7	47.1
8-10 years	46.2	40.2	45.1	40.9
>10 years	21.1	11.8	22.3	12.1
Smoking (%)				
Never	35.4	4.8	34.9	5.1
Former	28.9	18.0	29.3	18.1
Current <15 g/d	13.0	19.0	13.5	19.5
Current 15-25 g/d	16.0	40.4	15.6	39.9
Current >25 g/d	6.8	17.7	6.7	17.5
Physical activity (%)				
Inactive	10.8	16.3	11.0	16.8
Moderately inactive	30.4	32.0	30.3	32.4
Moderately active	24.2	21.2	23.7	20.5
Active	34.7	30.5	35.0	30.2
Waist circumference (cm)[1]	89.0	91.3	90.0	92.0
Body mass index (kg/m^2)[1]	25.5	25.5	25.8	25.6
Alcohol intake (g/d)[1]	12.9	16.5	13.8	17.0
Co-morbidities (%)				
Hypercholesterolemia	7.4	13.8	8.0	13.8
Hypertension	16.1	27.8	15.9	27.9
Diabetes mellitus	2.0	10.7	2.0	9.9

[1] Median

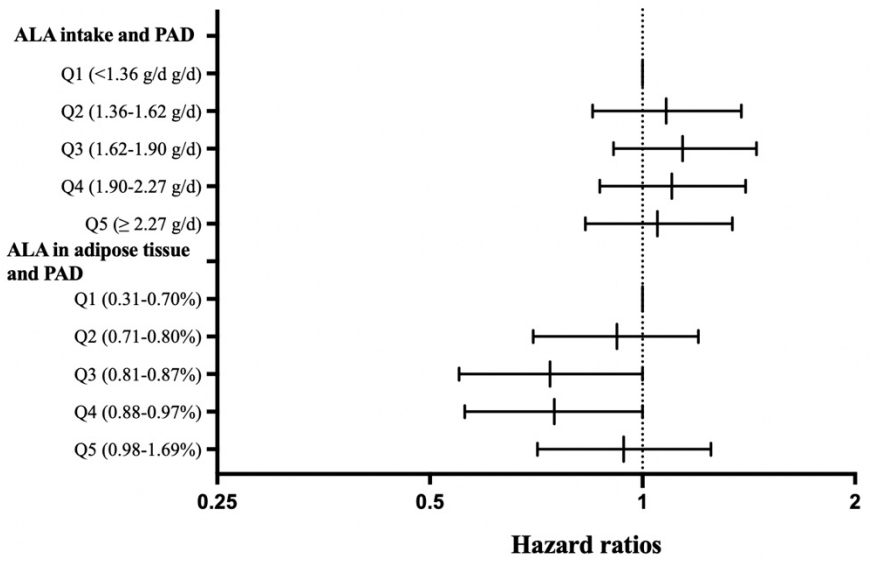


Figure 8. Forest plot of the associations between quintiles of ALA and the rate of PAD in analyses including adjustment for established risk factors (model 1B).

Conclusions

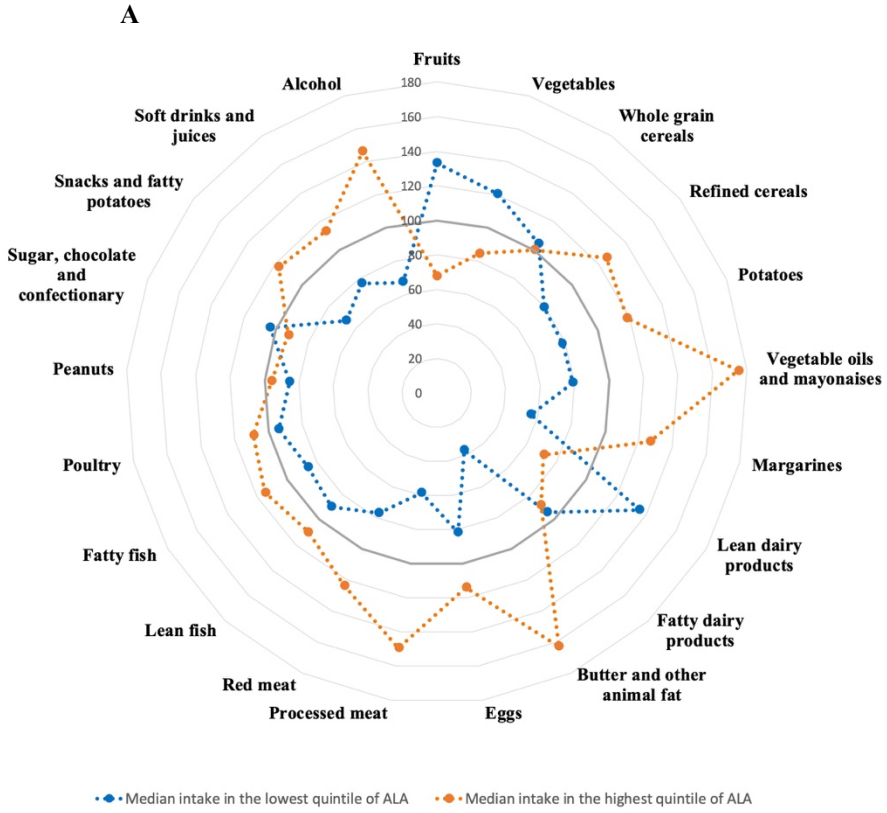
ALA intake was not appreciably associated with the rate of PAD. Indications of a U-shaped association between adipose tissue content of ALA was observed, but the association were not statistically significant.

5.5 ALA AND THE UNDERLYING DIETARY PATTERN

We used radar plots to graphically describe our exposures of interest as indicators of an underlying dietary pattern.

Participants in the highest quintile of energy-adjusted ALA intake had higher intakes of vegetable oils and mayonnaises, margarines, butter and other animal fat, eggs, processed and red meat, fish, poultry, snacks and fatty potatoes, soft drinks and juices, alcohol, refined cereals and potatoes, and lower intakes of fruits, vegetables and dairy products (Figure 9A).

Participants in the highest quintile of adipose tissue content of ALA within the sub-cohort had higher intakes of vegetable oils and mayonnaises, margarines, butter and other animal fat, processed meat, fish and refined cereals, and lower intakes of fruits, vegetables, dairy products, peanuts, snacks and fatty potatoes, soft drinks and juices and alcohol (Figure 9B).



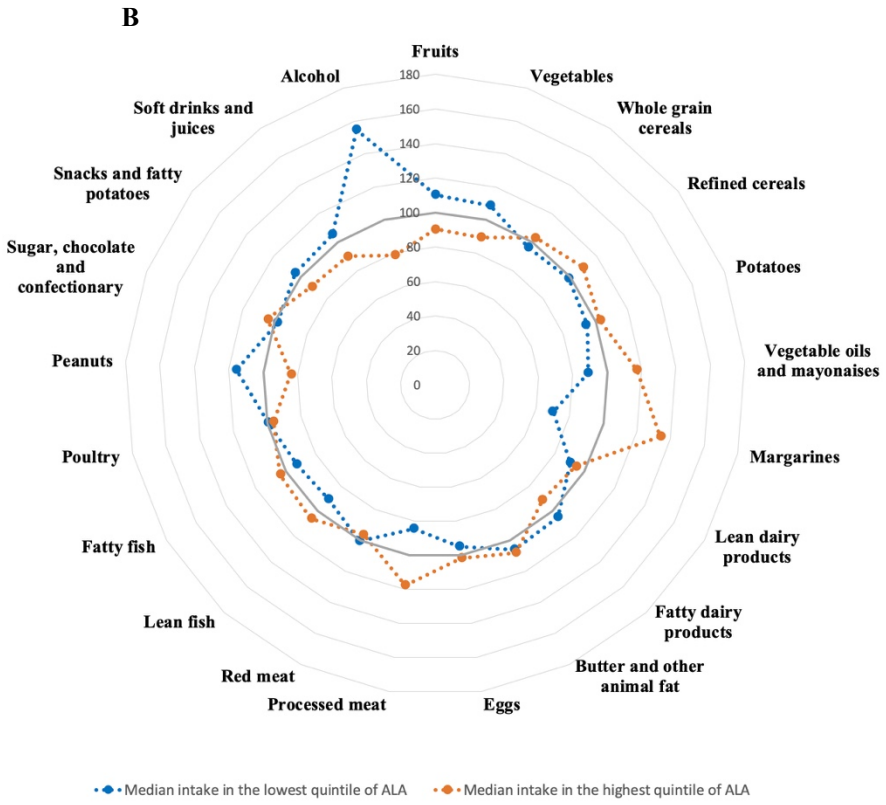


Figure 9. Radar plot of the median intake of selected foods in the highest (•◆•) and lowest (•◆•) quintile of ALA intake in the cohort (A) or quintile of adipose tissue content of ALA in the sub-cohort (B) indexed according to the median in the study populations described in paper IV and paper V, respectively.

CHAPTER 6. DISCUSSION

We found that dietary intake of ALA was not consistently nor appreciably associated with the rate of incident MI, total ischemic stroke or ischemic strokes of presumed atherosclerotic origin or with PAD. However, we did observe a U-shaped pattern of associations between ALA content in adipose tissue and the rate MI in women and between ALA content in adipose tissue and the rate of total ischemic stroke, ischemic stroke due to large artery atherosclerosis and PAD although only statistically significant in analyses of ischemic stroke due to large artery atherosclerosis. Overall our studies therefore did not lend support to our hypothesis that our estimates of ALA exposure were inversely associated with incident MI, ischemic stroke or PAD.

6.1 STRENGTHS AND LIMITATIONS

The strength and limitations of the studies included in this thesis have been described in detail in the individual papers, but some issues will be discussed below.

Selection problems

Selection bias may arise from procedures used to select participants and from factors that may influence study participation when related to both exposure and outcome of interest. Selection bias is, however, not likely to be a major concern in our studies because information on exposures was collected independently of the outcomes of interest and all cases were ascertained using nationwide registers with very limited loss to follow-up.

Information problems

Information problems may arise from inaccurate assessment of the exposures and/or misclassification of the outcomes of interest.

Information on dietary intake of ALA is likely to have been subject to measurement error as we relied on information on diet obtained through a single FFQ designed to assess the average intake of selected foods and beverages during the previous year. Further, calculation of estimated ALA intake based on specified portion sizes and the use of food composition tables may have contributed with error. Measurement error of dietary exposures is inevitable although it may be reduced using analytical approaches such as energy-adjustment (54). The FFQ used in the DCH cohort has been validated against two times 7-days weighted diet records and found suitable to categorise subjects according to their intake of energy and PUFAs, but intake of ALA was not specifically validated (45). However, measurement error in the assessment of

ALA intake probable occurred at random as information on diet was collected independently of the outcomes of interest. Random measurement error generally leads to attenuation of observed associations and loss of statistical power. Indeed, repeated dietary measurements would have been preferable in order to reduce random measurement errors and to capture potential changes in dietary habits during follow-up. However, the content of ALA in human tissue may represent an objective marker of intake that may be less prone to measurement error. The use of biomarkers as measures of exposure may be of particular importance to reflect ALA exposure, because sources rich in ALA such as some plant oils and margarines are used in food preparation and therefore may be difficult to report (11). Adipose tissue content of ALA is considered the gold standard to reflect the long-term exposure of ALA (64,65), but the content of ALA in adipose tissue may be influenced by metabolism and intake of other fatty acids. However, the content of ALA in adipose tissue may provide an estimate of the endogenous exposure, which may be more important from a biological point of view.

Confounding

We included detailed adjustment for potential risk factors defined prior to data analysis, but residual confounding arising from inaccurate self-reported information on covariates, insufficient adjustment and risk factors not taken into account cannot be ruled out in our analyses.

We applied adjustment for risk factors in different models. In general, the observed associations for each outcome in the minimally adjusted models taking into account age and sex (1A) were weakened after additional adjustment for established risk factors (model 1B), which might indicate confounding from these factors. Additional adjustment for comorbidities (model 2) showed overall similar pattern of associations compared with results obtained from analyses including adjustment for established risk factors (model 1B). This might suggest that residual confounding from the included comorbidities (model 2) might not be of major importance although findings from these models should be interpreted with caution as the included comorbidities might be intermediates.

In supplemental analyses, we added potential dietary risk factors to model 1B in analyses of ALA intake (model 3). Overall similar pattern of associated compared to model 1B were observed. We did not have information on intake of trans fatty acids, which would have been preferable as some early studies investigating ALA intake in relation to risk of CHD reported positive correlations between intake of ALA and trans fatty acid intake (33,66). However, the content of trans fatty acids in margarines, an important source of ALA, was reduced by more than 50% in the early 1990s in Denmark, and in the late 1990s trans fatty acids were practically absent in Danish margarines (67). Nevertheless, interpretation measures of associations obtained from analyses including adjustment for dietary factors should be interpreted with great

caution because fatty acids may share food sources and adjustment for dietary factors may introduce restrictions in the underlying dietary pattern that are not comparable with the underlying dietary pattern explored in model 1B.

We used radar plots to describe dietary intake and adipose tissue content of ALA as indicators of an underlying dietary pattern and to evaluate potential confounding from the underlying dietary pattern. These radar plots indicated that the energy-adjusted intake of ALA, but also adipose tissue content of ALA, were indicators of complex underlying dietary patterns. However, the radar plots did not indicate that a high intake or adipose tissue content of ALA reflected overall healthy dietary patterns.

Generalizability

The generalizability of the findings in this thesis may be limited according to the eligibility criteria for being invited to participate in the DCH cohort study and the exclusion criteria applied to the study populations under study. Thus, our findings may not be generalized to individuals who have not survived until the age of 50 years without a previous diagnosis of cancer and the outcome of interest in each study. Also, the participants enrolled into the DCH cohort was recruited from selected areas in Denmark and included almost exclusively Causations. In addition, only 35% of those invited to participate in the DCH cohort agreed to participate and those enrolled had a higher socioeconomic position compared with invited non-participants (43).

6.2 ALA AND SUGGESTED MECHANISMS OF ACTION

We studied the incorporation and metabolism of ALA and LA cultured in EA.hy926 endothelial cells and the production of inflammatory mediators when exposed to ALA, LA or different concentrations of ALA and LA followed by stimulation with tumour necrosis factor alpha (68). In these in vitro experiments, we found that ALA was incorporated into endothelial cells and that cells incubated with ALA had a higher absolute content of eicosatetraenoic acid, EPA and DPA, but not DHA, compared to endothelial cells cultured in culture medium (68). Further, the medium of endothelial cells incubated with ALA had a lower concentration of vascular endothelial growth factor, regulated on activation normal T cell expressed and secreted, intercellular adhesion molecule-1, monocyte chemoattractant protein-1 and interleukin-6, but not interleukin-8, when comparing with endothelial cells cultured in culture medium (68). These findings suggest that ALA can be further elongated in EA.hy926 endothelial cells and that incubation with ALA may lower various aspects of inflammation in these cells.

In humans, ALA intake has also been ascribed anti-inflammatory properties although results have not been consistent (11,12). Several cross-sectional studies have reported

inverse associations between ALA intake or content of ALA in blood components and plasma levels of C-reactive protein (69–73), IL-6 (69) and its soluble recepter (74), IL-1ra (73), soluble vascular cell adhesion molecule-1 (75) and E-selection (69,75). Also, intervention studies have suggested that supplementation with ALA or foods rich in ALA may lower aspects of inflammation (76–81).

Several studies investigating increased ALA consumption on blood lipid concentrations have suggested that ALA may have a LDL-cholesterol lowering effect, but studies have shown inconsistent findings and effects on triglycerides and other lipoproteins are less clear (12). Also, studies investigating increased ALA consumption on blood pressure have shown conflicting results, but few studies have reported modest inverse associations between ALA exposure and blood pressure (12). Also, ALA has been hypothesized to lower platelet aggregation (11,82). Furthermore, few follow-up studies have reported inverse associations between ALA intake and circulating levels of ALA and the risk of incident type 2 diabetes mellitus (83–85). Thus, several potential health benefits of ALA exposure have been suggested that might contribute to reduced atherosclerosis. Interestingly, studies have supported that ALA intake or content in serum and erythrocytes may be inversely associated with carotid intima media thickness and plaque burden in the carotid and femoral arteries (86–88).

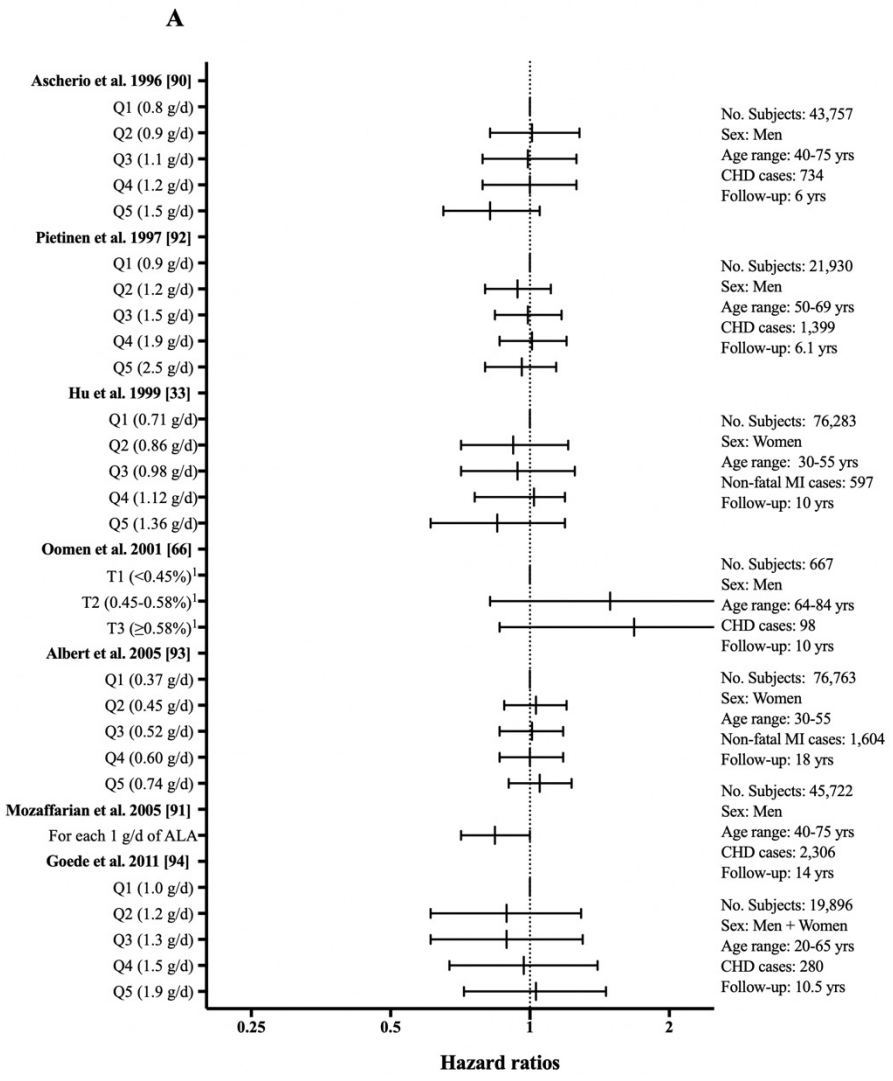
6.3 OBSERVATIONAL STUDIES OF AN ASSOCIATION BETWEEN ALA AND ASCVD

Several follow-up studies have investigated the association between dietary intake of ALA and CHD (10) (Figure 10). A possible association with ischemic stroke has been less studied (89) and no reported follow-up study has investigated associations between ALA intake and the rate of ischemic stroke subtypes or PAD. Also, no reported follow-up studies have reported on an association between adipose tissue content of ALA and the risk of MI, ischemic stroke or PAD.

ALA and CHD

Early findings reported by Ascherio et al. (90) based on data from the US Health Professionals Follow-up study suggested indications of a lower rate of incident CHD (non-fatal MI and fatal CHD) when comparing the highest quintile of ALA intake with the lowest, but no consistent pattern of association was observed across quintiles. A statistically significant inverse association was observed when ALA was expressed as a percentage of total energy intake in continuous linear analyses including adjustment for traditional risk factors and potential dietary risk factors (90). An

updated report from the same population by Mozaffarian et al. (91) after 14 years of follow-up found a lower rate of CHD with higher ALA intake in subjects with a low intake of EPA+DHA (<100 mg/day), while no association was observed among subjects with a higher intake of EPA+DHA (≥100 mg/day). The median intake of EPA+DHA intake within the DCH cohort was, however, markedly higher than in the Health Professionals Follow-up study and stratified analyses with a sufficient statistical power at such low levels was not possible.



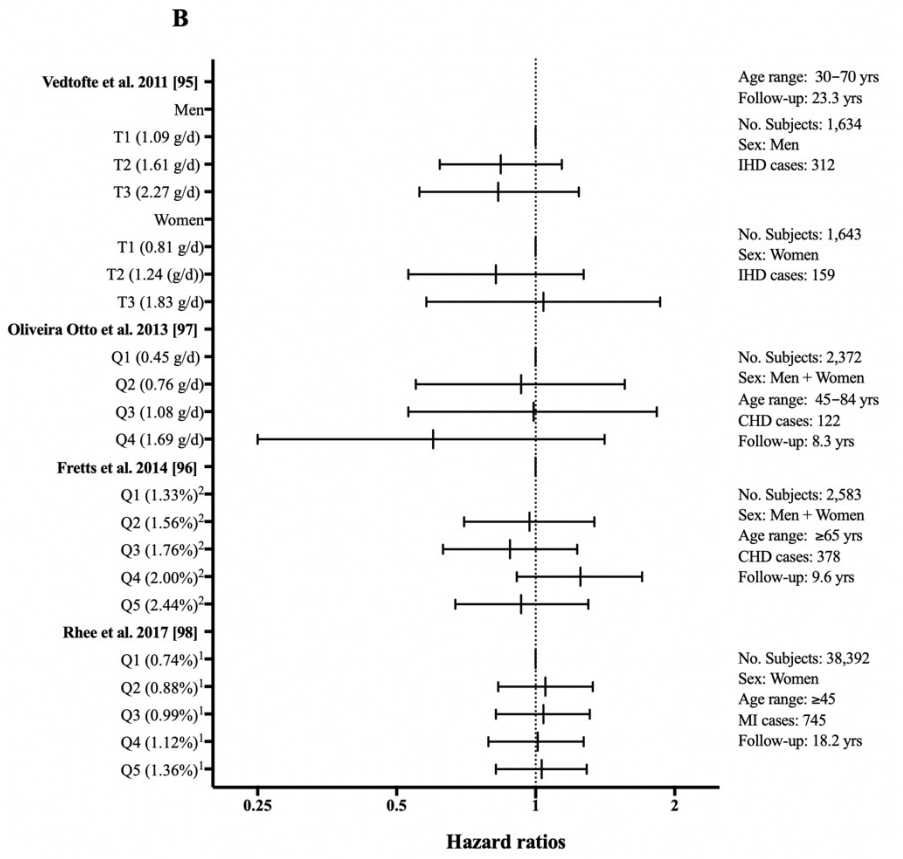


Figure 10A+B. Follow-up studies investigating ALA intake and incident CHD. Presented point estimates were selected from models that included adjustment for risk factors without including dietary risk factors whenever possible.
[1] ALA intake as a percentage of total energy intake; [2] ALA intake as a percentage of total fatty acids

Several other studies have not confirmed inverse association between ALA intake and the rate of CHD (33,66,92–98). Also, a pooled analysis by Vedtofte et al. (99) including data from eight American and European cohorts did not find ALA intake associated with the rate of total CHD after adjustment for traditional risk factors. However, in analyses including adjustment for traditional risk factors and dietary risk factors indications of lower rates of total CHD and fatal CHD in men were observed, whereas no appreciable association was observed amongst women (99).

Some studies have reported inverse or indications of inverse associations between ALA intake and fatal CHD (33,98–101), but the results have not been consistent (33,66,92,93,96,98–101). In supplemental analyses, we observed an inverse U-shaped association between ALA intake and fatal MI and a J-shaped association between adipose tissue content of ALA and fatal MI among men, but these associations were not statistically significant. Interestingly, a study by Albert et al. (93) observed lower rates of sudden cardiac death with higher ALA intake and suggested that ALA may influence cardiovascular risk predominantly through effects on fatal arrhythmias as no associations was observed between intake of ALA and non-fatal MI or other fatal CHD events.

Limited knowledge is available on the association between adipose tissue content of ALA and CHD, but a case-control study from Costa Rica by Campos et al. (102) reported a statistically significant inverse association between adipose tissue content of ALA and the odds of non-fatal MI. However, findings from other case-control studies investigating adipose tissue content and the odds of MI have not been consistent (103–105) (Figure 11).

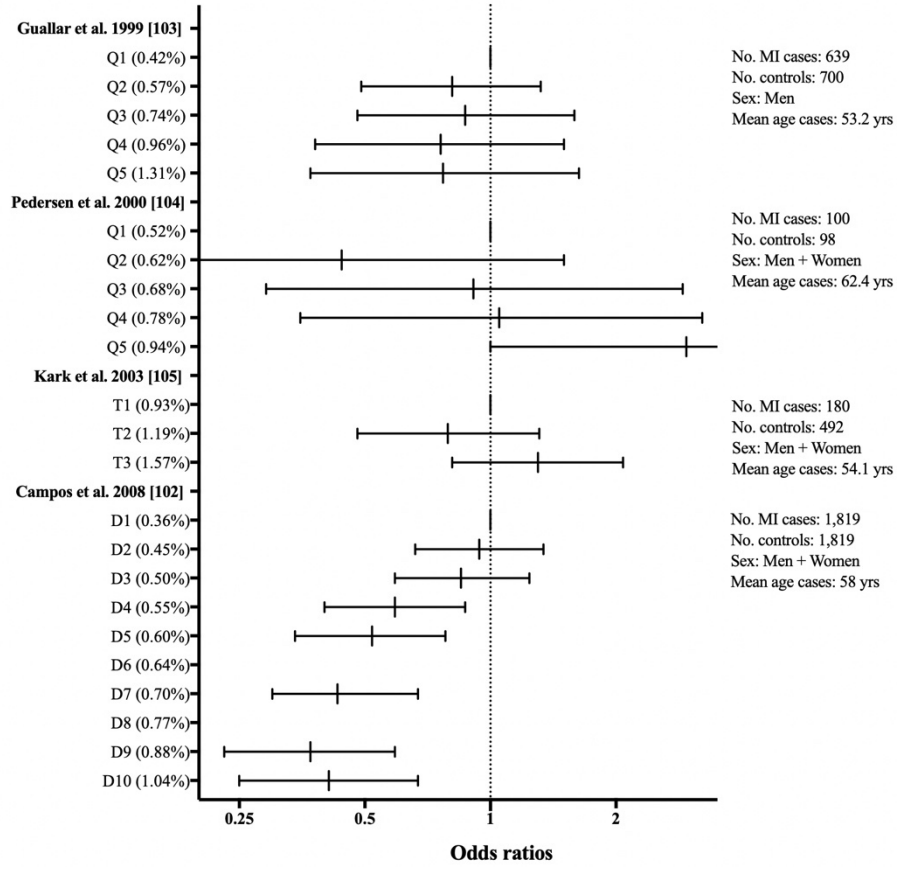


Figure 11. Case-control studies investigating adipose tissue content of ALA and MI. Presented point estimates were selected from models that included adjustment for risk factors without including dietary risk factors whenever possible. All studies expressed adipose tissue content as a percentage of total fatty acids.

A pooled analysis by Gobbo et al. (106) did not find biomarkers of ALA including the content of ALA in plasma, phospholipids, cholesterol esters and adipose tissue associated with total CHD (6,960 cases), but reported a modest inverse association between biomarkers of ALA and fatal CHD (2,640 cases). However, a Swedish follow-up study found no association between adipose tissue content of ALA and the rate of cardiovascular death (107).

In summary, follow-up studies investigating the association between ALA intake and incident CHD have shown conflicting results, but most studies have not supported an inverse association. However, ALA intake may be associated with a lower risk of

CHD in subjects with a low intake of LC n-3 PUFAs although this hypothesis warrants further investigation. Studies investigating biomarkers of ALA exposure have also shown conflicting results.

ALA and ischemic stroke

Few studies have investigated an association between ALA intake and the rate of total ischemic stroke (94,96,98,108) (Figure 12). Interestingly, a study by Goede et al. (94) observed lower rates of total ischemic stroke across quintiles of ALA and a study by Fretts et al. (96) found an indication of lower rates of total ischemic stroke when comparing the highest quintile of ALA intake with the lowest although no consistent pattern of association was observed across quintiles. In contrast, two other follow-up studies supported our findings of no association between ALA intake and the rate of total ischemic stroke (98,108). Also, most nested case-control studies investigating ALA content in blood components do not support that ALA exposure is consistently nor appreciably associated with the risk of total ischemic stroke (96,109–113). Previous follow-up studies have not investigated intake or adipose tissue content of ALA in relation to ischemic stroke subtypes. However, a nested case-control study by Iso et al. (109) reported lower odds of lacunar infarctions and large artery occlusive infarctions with higher ALA content in serum, but these associations were not statistically significant and detailed adjustment for risk factors were not undertaken.

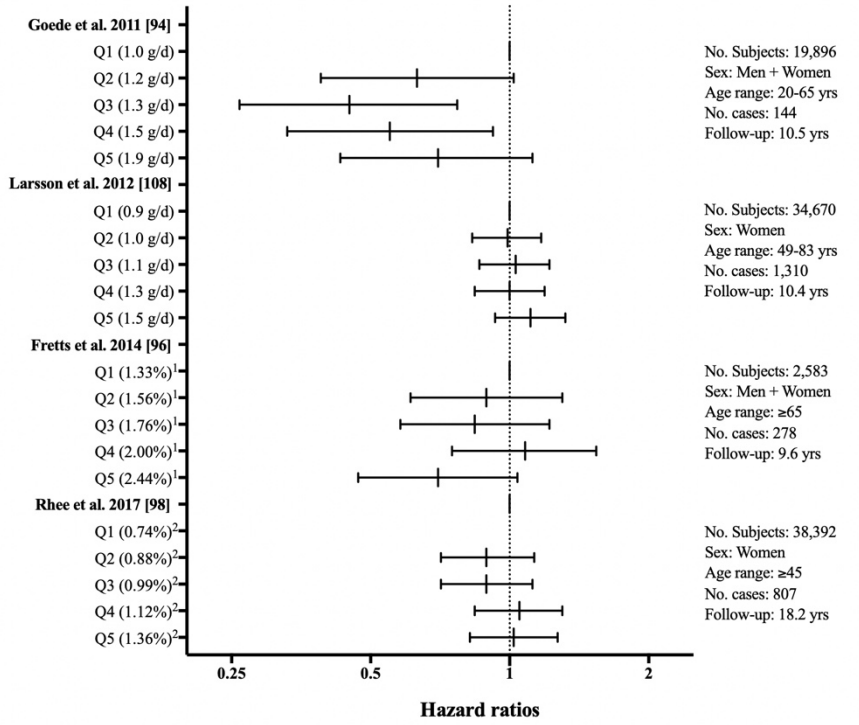


Figure 12. Follow-up studies investigating ALA intake and total ischemic stroke. Presented point estimates were selected from models that included adjustment for risk factors without including dietary risk factors whenever possible.
[1] ALA intake as a percentage of total fatty acids; [2] ALA intake as a percentage of total energy intake

ALA and PAD

To our knowledge, no previous follow-up studies have investigated the association between dietary intake or adipose tissue content of ALA and the rate of PAD. However, a cross-sectional study reported lower odds of lower limb disease with ALA content in erythrocytes (114), but detailed adjustment for risk factors were not performed.

6.4 CLINICAL SUPPLEMENTATION TRIALS WITH ALA

Few clinical trials have investigated the effect of ALA supplementation of or foods rich in ALA on vascular outcomes.

In a Norwegian trial (115), 13,406 men aged 50 to 59 years were randomised to receive either 10 mL of refined linseed oil (rich in ALA) or sunflower oil (rich in LA) both supplemented with vitamin E (116). The subjects were followed for one year and no difference in the number of incident MIs, fatal CHD or deaths were observed between the two groups (115). However, this study was initiated in 1965 and was hampered by few cases and a poor compliance to the supplement regimens.

In the randomized single-blinded Lyon Diet Heart Study (14), 605 patients aged less than 70 years of age with a prior MI were randomly assigned to either a Mediterranean-type diet supplemented with an ALA-rich margarine based on canola oil (providing 1.5 g/d of ALA) or a prudent Western diet (controls) (14,117). Patients assigned to the active group also received dietary counselling including advice to use canola oil and olive oil for salads and food preparation, to eat more bread, root vegetables, green vegetables, fish and fruits, and to lower their intake of red meat by replacing it with poultry and lower their intake of butter and cream by use of the ALA-rich trial provided margarine (14). After 27 months of follow-up, an intermediate analysis showed a substantially lower rate (HR: 0.27, 95% CI: 0.12-0.59) of recurrent MI and cardiac deaths in subjects assigned to the active group compared to controls and the trial was stopped. An updated report with extended follow-up for 46 months confirmed the initial findings (117), however, few events occurred in both treatment arms and given the nature of the multicomponent intervention the observed lower rate of MI and cardiac death can not be specifically attributed to a higher ALA intake.

In the double-blinded, more recent placebo-controlled Alpha-Omega-Trial (118), 4,837 patients aged 60 to 80 years of age with a prior MI were randomly assigned to receive trial margarines providing either 2g ALA, 0.4g EPA+DHA or both, or placebo (margarine without n-3 supplementation) according to a parallel 2-by-2 factorial design. Those assigned to ALA supplementation were compared to subjects assigned to placebo and EPA+DHA rather than placebo alone. After 40 months of follow-up, a modest statistically non-significant lower rate of major cardiovascular events (HR: 0.91, 95% CI: 0.78-1.05) including fatal and non-fatal cardiovascular events and cardiac interventions were observed in subjects assigned to ALA supplementation (319 events) compared to those assigned to placebo or EPA+DHA only (352 events) (118). In subgroup analyses, statistically non-significant lower rates of major cardiovascular events were observed in subjects below 70 years of age (HR: 0.83, 95% CI: 0.67-1.03) and in women (HR: 0.73, 95% CI: 0.51-1.03) receiving supplementation with ALA compared to those receiving EPA+DHA supplementation and placebo. No associations were observed in males and subjects above 70 years of age (118). These subgroup analyses should, however, be interpreted with caution due to a limited number of events.

The large methodological heterogeneity in terms of study populations and designs, composition of supplementations, and trial duration makes comparison of study findings from these clinical supplementation trials difficult. Randomized controlled trials with hard endpoints are generally considered the gold standard of scientific evidence. However, in the context of nutritional research carefully conducted prospective observational studies may be more suited to answer questions on the role of dietary exposures in the general population on development of ASCVD because the etiologic period is long and large clinical supplemental trials are seldom feasible in a setting of primary prevention.

CHAPTER 7. CONCLUSIONS AND PERSPECTIVES

In this thesis associations between dietary intake of ALA and adipose tissue content of ALA and development of ASCVD including MI, ischemic stroke and PAD were investigated. We found that dietary intake of ALA was not consistently nor appreciably associated with the rate of incident MI, total ischemic stroke or ischemic strokes of presumed atherosclerotic origin or PAD. We did, however, find a statistically significant U-shaped association between the content of ALA in adipose tissue and the rate of ischemic stroke due to large artery atherosclerosis and indications of U-shaped pattern of associations between ALA content in adipose tissue and the rate of MI in women, total ischemic stroke and PAD.

The observed U-shaped pattern of associations in analyses of adipose tissue content of ALA for these different atherosclerotic outcomes included in this thesis is not clear, but might impose that metabolic factors may be of importance for the association between ALA exposure and development of ASCVD. Thus, ALA may be influenced by several factors from intake to incorporation into adipose tissue including competition between PUFAs for metabolism and incorporation into adipose tissue and potential genetic differences in the shared enzymes responsible for PUFA synthesis. Therefore, further research on whether interplay between PUFAs and potential genetic differences in shared metabolic enzymes influence the associations between PUFA intake and development of ASCVD is warranted.

Studies relying on self-reported intake may be limited by measurement error, which may reduce the possibility to detect true associations. In contrast, the content of ALA in human tissue such as adipose tissue can be assessed more precise and the content herein are considered an objective long-term biomarker of endogenous ALA exposure. However, the content of ALA in human tissue may be influenced by metabolism and intake of other fatty acids. Thus, both dietary and biomarker studies of ALA do have limitations, but the use of complementary estimates of exposure may contribute with valuable information and should be considered when possible in future studies. However, it should be emphasized that our estimates of exposure were indicators of an underlying dietary pattern. We described these dietary patterns using radar plots and found that our measures of exposures were indicators of complex underlying dietary patterns, which may differ by country and the population under study. Interestingly, ALA has been suggested to be an important part of the Mediterranean diet, which has been associated with a lower risk of major cardiovascular events (15). However, the protective effect on CVD provided by the Mediterranean diet may be attributable to a combination of several dietary factors acting in concert rather than a single component like ALA. Thus, it should be stressed that we did not evaluate the potential effect of a Mediterranean diet on ASCVD risk,

but the associations between estimates of ALA exposure representing indicators of underlying dietary patterns in relation to development of ASCVD. However, investigation of dietary patterns such as the Mediterranean diet in relation to development of ASCVD represent interesting areas for further research. Dietary pattern analyses may circumvent challenges in differentiating between individual and correlated dietary factors and may address the combined effect of dietary patterns on disease risk, which may be of great public health relevance. Further, the associations between PUFAs and ASCVD may depend on the nutrients used to replace them and substitution aspects of dietary factors in relation of ASCVD risk warrant further investigation.

ENGLISH SUMMARY

Atherosclerotic cardiovascular disease (ASCVD) remains the leading cause of death globally. The plant-derived n-3 polyunsaturated fatty acid (PUFA), alpha-linolenic acid (ALA) has been associated with beneficial effects on atherosclerosis development, but previous investigations on hard vascular endpoints have shown inconsistent results. Further, most previous studies have focused on coronary heart disease, while the other major atherosclerotic diseases including ischemic stroke and PAD have been less studied.

The objective of this thesis was to investigate the associations between the energy-adjusted intake of ALA and adipose tissue content of ALA and the rate of myocardial infarction (MI), ischemic stroke and peripheral artery disease (PAD). We hypothesized that these estimates of ALA exposure were inversely associated with the rate of incident MI, ischemic stroke and PAD.

This thesis was based on data from the Danish Diet, Cancer and Health cohort, which is a cohort of more than 57,000 middle-aged Danish men and women. Intake of ALA was assessed using a food frequency questionnaire. Adipose tissue content of ALA was determined by gas chromatography in all cases and a randomly drawn sub-cohort. All cases were identified by record linkage with nationwide Danish registers. The association between energy-adjusted ALA intake and the outcomes of interest were investigated using a traditional cohort design, whereas the association between adipose tissue content of ALA and outcomes were investigated using a case-cohort design. Hazard ratios obtained using Cox proportional hazard regression were used as measures of association.

We found that dietary intake of ALA was not consistently nor appreciably associated with the rate of incident MI, total ischemic stroke or ischemic strokes of presumed atherosclerotic origin or PAD. In analyses of adipose tissue content of ALA, we observed indications of U-shaped pattern of associations between ALA content in adipose tissue and the rate of MI in women, total ischemic stroke and PAD, but these associations were not statistically significant. However, in analyses of ischemic stroke subtypes of atherosclerotic origin we observed a statistically significant U-shaped association between adipose tissue content of ALA and ischemic stroke due to large artery atherosclerosis, while no appreciable association was observed for ischemic strokes due to small-vessel occlusion.

In conclusion, our studies did not lend support to our hypothesis that estimates of ALA exposure were inversely associated with the risk incident MI, ischemic strokes or PAD. Perspectives for further research on the association between ALA and ASCVD include investigations of the influence of potential interaction between ALA and the major n-6 PUFA linoleic acid on ASCVD risk and the importance of genetic differences in the shared metabolic enzymes important for PUFA metabolism on ASCVD risk warrant further investigation.

DANSK RESUME

Aterosklerotisk kardiovaskulærsygdom er fortsat den primære årsag til død på verdensplan. Den plante-derivede n-3 polyumættede fedtsyre alpha-linolensyre (ALA) er blevet forbundet med gunstige effekter som menes at reducere åreforkalkning, men tidligere studier med hårde vaskulære endepunkter har vist inkonsistente resultater. Ydermere har de fleste tidligere studier fokuseret på koronarkarsygdom, mens sammenhængen mellem ALA og iskæmisk slagtilfælde og perifer arteriesygdom kun i begrænset omfang er blevet undersøgt.

Formålet med denne afhandling var at undersøge sammenhængen mellem det energi-justerede indtag af ALA samt indholdet af ALA i fedtvæv og risikoen for udvikling af myokardieinfarkt, iskæmisk slagtilfælde og perifer arteriesygdom. Vores hypotese var at disse mål for eksponering af ALA var inverst forbundet med risikoen for udvikling af myokardieinfarkt (MI), iskæmisk slagtilfælde og perifer arteriesygdom.

Denne afhandling er baseret på data fra den danske befolkningsundersøgelse Kost, Kræft og Helbred, som er en kohorte med mere end 57,000 midaldrende danske mænd og kvinder. Indtaget af ALA blev beregnet med udgangspunkt i et fødevare-frekvensspørgeskema, mens indholdet af ALA i fedtvæv blev bestemt med udgangspunkt i gaskromatografi blandt alle cases og en vilkårligt udtrykket sub-kohorte. Alle cases blev identificeret gennem nationale danske registre. Sammenhængen mellem det energi-justerede indtag af ALA og de tidligere beskrevne udfald blev undersøgt med udgangspunkt i et traditionelt kohortedesign, mens analyser baseret på indholdet af ALA i fedtvæv blev undersøgt baseret på et case-kohortedesign. Hazard ratioer beregnet med udgangspunkt i Cox proportional hazard regression blev benyttet som associationsmål.

Vi fandt ingen sammenhæng mellem indtag af ALA og risikoen for udvikling af MI, iskæmisk slagtilfælde og perifer arteriesygdom. I analyser af indholdet af ALA i fedtvæv fandt vi indikationer på en U-formet sammenhæng mellem indholdet af ALA i fedtvæv og risikoen for MI blandt kvinder, total iskæmisk slagtilfælde og perifer arteriesygdom. I analyser af subtyper af iskæmisk slagtilfælde fandt vi en statistisk signifikant U-formet sammenhæng mellem indholdet af ALA i fedtvæv og risikoen for iskæmisk slagtilfælde forårsaget af storkarssygdom, mens ingen betydende sammenhæng blev fundet for iskæmisk slagtilfælde forårsaget af småkarssygdom.

Disse studier bekræfter således ikke vores hypotese om, at indtag eller koncentrationen af ALA i fedtvæv er inverst associeret med risikoen for udvikling af MI, iskæmisk slagtilfælde eller perifer arteriesygdom. Perspektiver for videre forskning i sammenhængen mellem ALA og aterosklerotisk kardiovaskulærsygdom inkluderer undersøgelser af betydningen af interaktion mellem ALA og den primære n-6 polyumættede fedtsyre, linolsyre, samt undersøgelser af betydningen af mulige genetiske forskelle i fælles enzymer involveret i omdannelsen af disse fedtsyrer.

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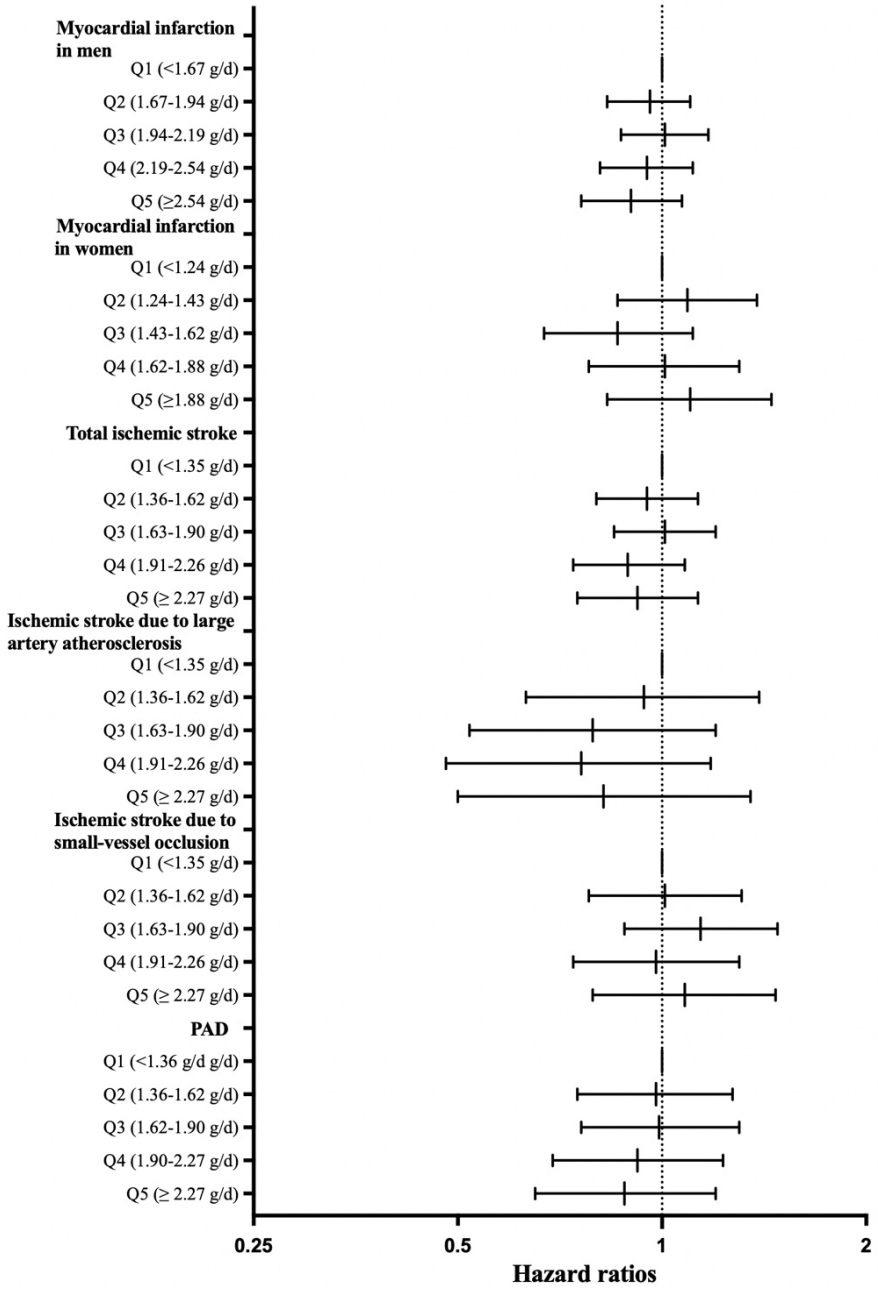
APPENDICES

Appendix Figure 1

Appendix Figure 1. Forest plot of the associations between quintiles of energy-adjusted ALA intake and the rate of MI, ischemic stroke and PAD. The analyses were adjusted for established risk factors and potential dietary risk factors (model 3).



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