N-6 AND MARINE N-3 POLYUNSATURATED FATTY ACIDS AND RISK OF ISCHEMIC STROKE.

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BY
STINE KROGH VENØ

DISSERTATION SUBMITTED 2019

AALBORG UNIVERSITY
DENMARK

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Stine Krogh Venø, MD

January 2019

ABBREVIATIONS

ALA	Alpha-linolenic acid
BMI	Body mass index
CI	Confidence interval
CHD	Coronary heart disease
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
HR	Hazard ratio
ICD	International Classification of Diseases
LA	Linoleic acid
LDL	Low-density lipoprotein
MUFA	Monounsaturated fatty acid
PUFA	Polyunsaturated fatty acid
Q	Quintile
SFA	Saturated fatty acid
TOAST	Trial of Org 10172 in Acute Stroke Treatment

LIST OF PAPERS

This thesis is based on the following four papers:

Paper I

Stine K. Venø, Erik B. Schmidt, Marianne U. Jakobsen, Søren Lundbye-Christensen, Flemming W. Bach, Kim Overvad. Substitution of Linoleic Acid for Other Macronutrients and the Risk of Ischemic Stroke.
***Stroke*. 2017;48:3190-3195.**

Paper II

Stine K. Venø, Christian S. Bork, Marianne U. Jakobsen, Søren Lundbye-Christensen, Flemming W. Bach, Kim Overvad, Erik B. Schmidt. Linoleic Acid in Adipose Tissue and Development of Ischemic Stroke: A Danish Case-Cohort Study.
J Am Heart Assoc. 2018;7:e009820.

Paper III

Stine K. Venø, Christian S. Bork, Marianne U. Jakobsen, Søren Lundbye-Christensen, Peter L. McLennan, Flemming W. Bach, Kim Overvad, Erik B. Schmidt. Marine n-3 polyunsaturated fatty acids and the risk of ischemic stroke.
***Stroke*. 2019;50:00-00.**

Paper IV

Stine K. Venø, Christian S. Bork, Marianne U. Jakobsen, Søren Lundbye-Christensen, Flemming W. Bach, Peter L. McLennan, Anne Tjønneland, Erik B. Schmidt, Kim Overvad. Substitution of Fish for Red Meat or Poultry and risk of Ischemic Stroke.
Nutrients 2018: 10111648

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CHAPTER 1. INTRODUCTION

Stroke is a major public health problem and cause of death. The majority of strokes are ischemic strokes that represent 80-90% of all strokes.[1]

Ischemic strokes often result in devastating and irreversible conditions and survivors of ischemic strokes may experience mental and physical impairment diminishing quality of life. Ischemic strokes place a substantial burden on families and health care systems and give rise to large societal costs.[2] Moreover, ischemic stroke increases with advancing age and the impact of ischemic stroke is expected to increase as the proportion of the aged population increases. In 2010, the global incidence of ischemic stroke was approximately 11.6 million events with almost 70% occurring in individuals >65 years of age.[1]

Almost 80% of ischemic strokes are first time diagnoses emphasizing the importance of primary prevention. Lifestyle and diet are the cornerstones of prevention and has the advantage that it is generally cheap and can be applied to whole populations and further adds to improvement of other health aspects. A key component of a healthy dietary pattern may be the intake of polyunsaturated fatty acids (PUFA).

This PhD thesis aimed to examine the association between long-term dietary intake of n-6 and marine n-3 PUFA and the risk of ischemic stroke and its subtypes. The thesis is based on four studies using data from the Diet, Cancer and Health cohort, which is a prospective cohort study of 57,053 Danish men and women.

CHAPTER 2. BACKGROUND

ISCHEMIC STROKE

Stroke is a major cause of death and long-term disability worldwide.[1] There are two main types of stroke; ischemic stroke and hemorrhagic stroke. These two types of strokes have entirely different etiology. Hemorrhagic stroke occurs when a blood vessel in the brain ruptures with subsequent bleeding into the brain. Ischemic stroke is caused by a blockage of an artery that supplies the brain resulting in inadequate supply of oxygen to the brain. Ischemic strokes accounts for 80-90% of all strokes in Western countries.[1]

Ischemic stroke is a heterogenous condition and can be divided into 5 subgroups according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification system,[3] which is a system based on etiology (Table 1).

***Table 1**: Subtypes of the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification system.*

Subtype	Diagnosis	Cause
Large artery atherosclerosis	>50 % stenosis or occlusion of a major brain artery or branch cortical artery of atherosclerotic origin.	Mainly due to atherosclerosis.[3]
Cardioembolism	Arterial occlusion due to an embolus arising in the heart and at least one cardiac source for an embolus must be identified	Mainly due to atrial fibrillation/atrial flutter.[4]
Small-vessel occlusion	Occlusion of small penetrating arteries providing blood to the deep structures of the brain. Large artery and cardiac sources must be excluded. The diagnosis is supported by a history of diabetes mellitus or hypertension.	Mainly due to lipohyalinosis or atherosclerosis.[5]
Stroke of other etiology	Clinical and CT or MRI findings of an ischemic stroke but blood tests or arteriography reveals a rare cause.	Strokes due to rare causes including: nonatherosclerotic vasculopathies, hypercoagulable states or hematologic disorders.[3]
Stroke of undetermined etiology	Ischemic stroke cases with incomplete evaluations or two or more potential causes.	

While ischemic strokes are of heterogenous origin, atherosclerosis is a common pathophysiological background behind many ischemic strokes. Atherosclerosis is a very complex process that occurs within the arterial wall due to a multifactorial life-long process, where lipids, inflammatory and hemostatic mediators lead to plaque formation.[6–9] The diameter of the artery shrinks with subsequent decrease of the blood flow resulting in reduced oxygen supply. Plaque rupture may lead to acute complications of atherosclerosis such as coronary heart disease (CHD) or ischemic stroke.[10,11] Ischemic stroke can also occur due to vessel occlusion by growth of a stable plaque, however, studies suggest that the predominant mechanism involves plaque rupture.[12–14]

Well established risk factors of ischemic stroke include age, sex, anthropometry, smoking, physical activity and alcohol intake.[1,2,15] Furthermore, some but not all studies have found an association between plasma cholesterol and risk of ischemic stroke.[16–21] Regarding lipids associations have mainly been attributed to plasma levels of low-density lipoprotein (LDL)-cholesterol and a meta-analysis of 14 randomized controlled trials found a lower risk of ischemic stroke when LDL-cholesterol was reduced.[22] The larger cerebral arteries may be more susceptible to LDL-cholesterol exposure, and therefore, LDL-cholesterol may be stronger associated with strokes due to large artery atherosclerosis.[23] Furthermore, plasma triglyceride levels may also be associated with ischemic stroke risk.[1,24]

Hypertension is the main risk factor for hemorrhagic stroke but is also a major risk factor of ischemic stroke and the risk increases progressively with increasing blood pressure.[15,25] Hence, randomized controlled trials have found a lower risk of stroke with reduction of blood pressure.[26,27]

Prospective cohort studies have reported positive associations between occurrence of diabetes mellitus and risk of ischemic stroke.[28] Subjects with diabetes mellitus are more prone to develop atherosclerosis and the prevalence of hypertension and hypercholesterolemia is increased in people with diabetes.[2]

Atrial fibrillation/atrial flutter is another important risk factor of ischemic stroke especially strokes due to cardioembolism.[29] Thus, studies have shown that chronic atrial fibrillation is associated with more than a fivefold higher risk of stroke.[30]

Diet may also influence the risk of ischemic stroke. In particular, some fatty acids from the diet may have detrimental effects on blood lipids, blood pressure, insulin sensitivity, arrhythmias, platelet aggregability, endothelial function and inflammation.[31–33]

FATTY ACIDS

Fatty acids can be classified as saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) or PUFA according to the number of double bonds (**Figure 1**). SFA lack double bonds, MUFA have one double bond whereas PUFA contain two or more double bonds (**Figure 2**).[34] SFA composes around 14 % energy of the average Danish dietary intake with butter, meat, sweet bakery products, confectionary and dairy products as the main sources.[35,36] MUFA is presented in several food groups and contributes with 13 % energy of the dietary intake.[35,36] The average Danish dietary intake of PUFA is 5.6 % energy and the main sources are soft margarines and vegetable oils.[35,36]

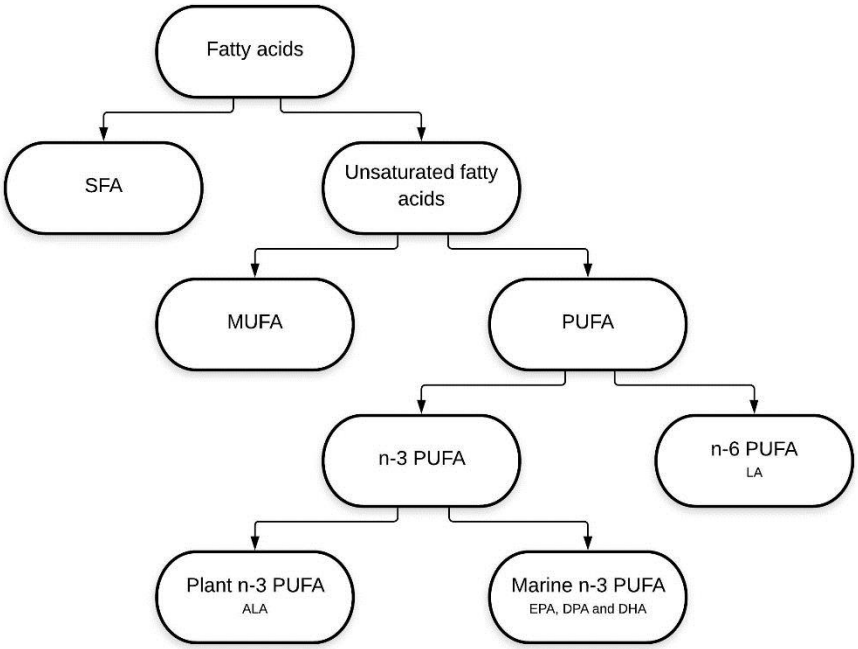

Figure 1: *Types of major dietary fatty acids. SFA indicates saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LA, linoleic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.*

PUFA from the diet can be divided into n-3 and n-6 PUFA. Counted from the methyl end, n-3 and n-6 have their first double bond in the n-3 position and n-6 position, respectively.

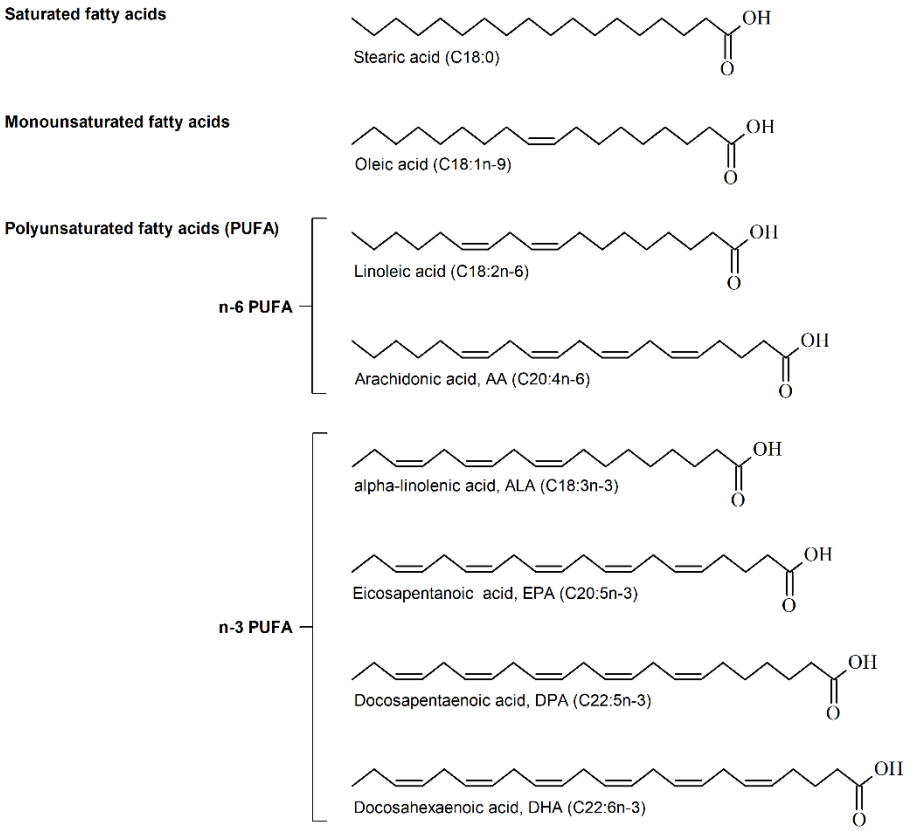

***Figure 2**: Classification of major fatty acids.*

N-3 PUFA can be divided into plant-derived n-3 PUFA, namely alpha-linolenic acid (ALA) and marine n-3 PUFA, such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA).

ALA can to a limited extent in humans be converted through a pathway involving desaturation to form stearidonic acid, and elongation to form eicosatetraenoic acid and further desaturation to form EPA (Figure 3). EPA can further undergo elongation, desaturation and oxidation to form DPA and DHA.[37–39] The main source of marine n-3 PUFA is seafood, especially fatty fish. The most widely consumed n-6 PUFA is linoleic acid (LA), which is derived from many different sources, although, vegetable oils is the primary source.[40–42] After consumption, LA can be converted to other n-6 PUFA by

steps similar to the conversion of ALA (Figure 3). LA is first desaturated to form gamma-linoleic acid, and elongated to form di-homo-gamma-linoleic acid and further desaturated to form arachidonic acid (AA).[43] AA can also be consumed in the diet with the most important dietary sources being eggs and meat.[44] LA and ALA are essential since they cannot be synthesized by the human body and must be provided in the diet.

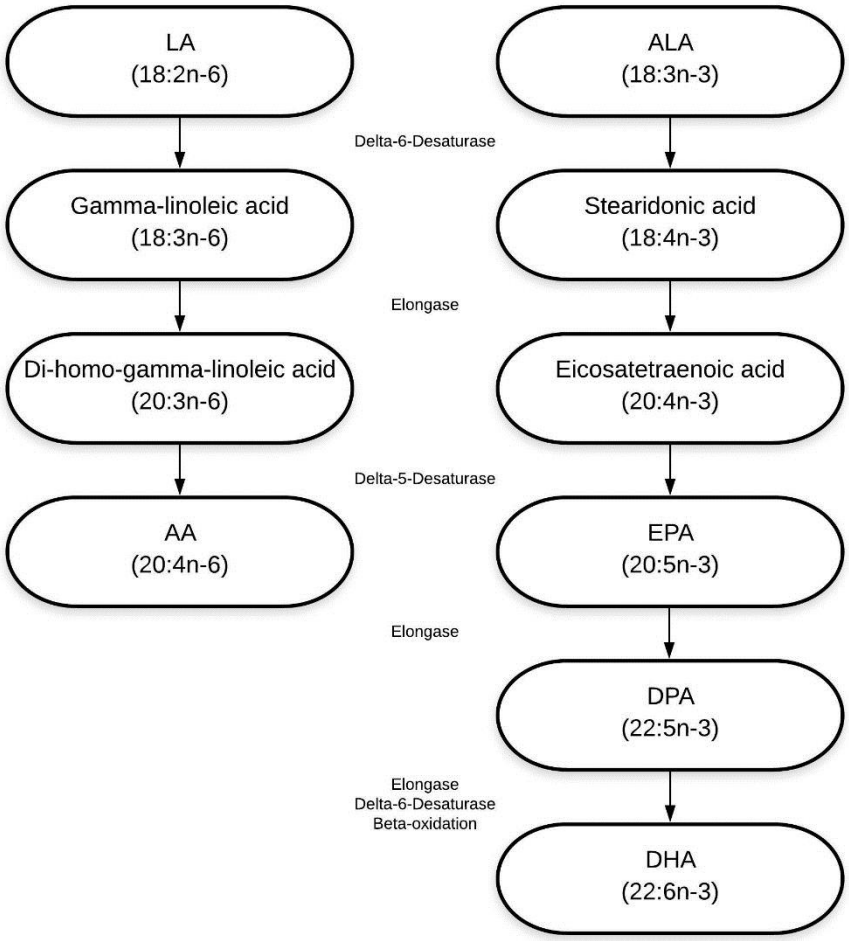

Figure 3: *Pathway of the conversion of LA to AA and ALA to DHA. LA indicates linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.*

N-3 and n-6 PUFA are incorporated into cells and tissues where they are stored and can be metabolized into biologically active and less active compounds. Thus, AA and EPA are incorporated into platelets and leucocytes from where they can be further metabolized into thromboxanes and leukotrienes, respectively.[43] Because AA can be converted to the proaggregatory and vasoconstrictive thromboxane A2 in platelets and proinflammatory leukotrienes B4 in leucocytes, it has been suggested that excessive LA intake could lead to increased thrombotic risk or inflammation and thereby to atherosclerotic diseases. EPA, however, is converted to the less proaggregatory thromboxane A3 and less proinflammatory leukotriene B5 suggesting beneficial effects of marine n-3 PUFA with respect to atherosclerosis and vascular diseases. Because of competition for the same enzymes it has been suggested that LA intake should be decreased while intake of EPA and DHA should be increased to reduce vascular disease.[45,46] However, because AA levels are under close homeostatic regulations, dietary intake of LA may not be correlated with levels of AA in plasma or adipose tissue.[43,47–49]

Marine n-3 PUFA have been associated with beneficial effects on blood pressure, plasma triglycerides, platelet aggregability and inflammatory measures.[39,50,51] LA has primarily been associated with a lowering of LDL-cholesterol, but LA may also lower blood pressure and improve insulin sensitivity.[52,53] Both LA and marine n-3 PUFA have shown inverse associations with CHD.[54,55] Though both CHD and ischemic stroke have atherosclerotic etiology, and despite beneficial associations with risk factors of ischemic stroke, prospective cohort studies of long-term intake of LA and marine n-3 PUFA in relation to ischemic stroke incidence have shown inconsistent results.

LA is being consumed in large quantities and contribute considerably to the total energy intake. Hence, if energy balance is maintained, a higher intake of LA must necessarily be accompanied by a lower intake of other macronutrients such as SFA, MUFA or glycemic carbohydrates. Similarly, intake of fish should be investigated as replacements of other food items.

CHAPTER 3. AIMS AND HYPOTHESES

The overall aim of this PhD thesis was to examine the association between long-term dietary intake of the n-6 PUFA LA and total marine n-3 PUFA, EPA and DHA and the risk of ischemic stroke and its subtypes.

The specific aims and hypotheses in this thesis were:

Study I

The aim of the first study was to investigate the hypothesis that a higher intake of LA and a concomitant lower intake of SFA, MUFA or glycemic carbohydrate was associated with a lower risk of ischemic stroke and its subtypes.

Study II

The aim of the second study was to investigate the hypothesis that adipose tissue content of LA was inversely associated with the risk of ischemic stroke and its subtypes.

Study III

The aim of the third study was to investigate the hypothesis that intake and content in adipose tissue of total marine n-3 PUFA, EPA and DHA were inversely associated with the risk of ischemic stroke and its subtypes.

Study IV

The aim of the fourth study was to investigate the hypothesis that a higher intake of fish and a concomitant lower intake of red meat or poultry was associated with a lower risk of ischemic stroke and its subtypes.

CHAPTER 4. METHODS

STUDY POPULATION

This thesis was based on data from the Danish Diet, Cancer and Health cohort study, which was originally established to investigate the role of diet and lifestyle in relation to incident cancer and other chronic diseases.[56] The study was initiated from December 1993 to May 1997 by inviting 160,725 men and women by mail. Non-responders received a second letter. A total of 57,053 men and women accepted the invitation corresponding to 35% of those invited. All participants were between 50-64 years old, citizens of the greater Copenhagen or Aarhus areas and not previously registered in the Danish Cancer Registry. All participants gave written informed consent at inclusion, and the study was approved by the relevant ethics committees and the Danish Data Protection Agency. A subcohort of 3500 participants was randomly drawn from the whole cohort. Participants with stroke or cancer or missing information on potential confounders before recruitment were excluded.

ASSESSMENT OF DIETARY INTAKE

At baseline participants filled in a validated semiquantitative food frequency questionnaire which included 192 food and drink items.[56,57] Participants were asked to indicate their mean intake of each item during the previous year. The predefined responses were reported in 12 categories ranging from never to more than 8 times/day. The average daily intakes of foods and nutrients was calculated using the FoodCalc program,[58] which is based on Danish food composition tables. At the study centres the food frequency questionnaires were optically scanned and checked for missing values and reading errors and uncertainties were checked by technicians with the study participants. The food frequency questionnaire was validated against two weeks of weighted diet records and correlations between energy adjusted intakes from the food-frequency questionnaire and the two-week diet records for PUFA were 0.53 for men and 0.28 for women.[59]

ASSESSMENT OF ADIPOSE TISSUE CONTENT OF FATTY ACIDS

All participants had an adipose tissue biopsy taken from the subcutaneous fat from the buttocks at baseline. A luer lock system (Terumo, Terumo Corp,

Tokyo, Japan) was used, which consisted of a needle, a venoject multi-sample luer adaptor and an evacuated blood tube according to the method of Beynen and Katan.[60] All adipose tissue samples were subsequently flushed with nitrogen and stored at -150 ^{o}C until analysis, where samples were thawed, and adipose tissue was removed to a glass and preheated at 50 ^{o}C for 10 min. Heptane was used to dissolve the fat at 50 ^{o}C and fatty acids were transesterified by 2 mol/L potassium hydroxide in methanol at 50 ^{o}C for 2 min, according to IUPAC standard methods for analysis of oils, fats and derivatives. Fatty acid composition of adipose tissue was determined by gas chromatography on a CP-sil 88 60 m×0.25 mm ID capillary column, consisting of a highly substituted, stabilized cyanopropyl stationary phase, using a Varian 3900 GC with a CP-8400 auto sampler (Varian, Middleburg, The Netherlands) equipped with a flame ionization detector. Commercially available standards (Nu-check-Prep, Inc., Min, US) were used to identify individual fatty acids and helium was used as the carrier gas. The contents of LA, EPA, DPA and DHA were given as weight percentage of total fatty acids. The inter-assay coefficient of variation for fatty acids in adipose tissue was 1.0% for LA, 6.1% for EPA, 4.2% for DPA and 5.2% for DHA.

ASSESSMENT OF COVARIATES

Participants filled in a lifestyle questionnaire at baseline regarding social factors, health status and lifestyle habits during the previous year. Education was reported as <7 years, 8 to 10 years, or >10 years. Information on physical activity was reported as number of hours per week spent on walking, biking, housework, home maintenance, gardening, and sports during summer and winter. Smoking habits during the past year were self-reported as frequency (never, former, or current), number, and type (cigarettes, cigars, cheroots, and tobacco pipes smoked per day). Hypercholesterolemia was also self-reported or defined by treatment with lipid-lowering agents. Similarly, data on hypertension was self-reported or defined by use of antihypertensive drugs. Information on diabetes mellitus was self-reported or defined by use of insulin. Information on atrial fibrillation/atrial flutter was found by linkage to the National Patient Register using International Classification of Diseases (ICD)-8 discharge codes 42793 or 42794 or ICD-10 discharge code I489.

Anthropometric measurements (height, weight, and waist circumference) were obtained by a technician at baseline. Body mass index (BMI) was calculated as weight (kg)/height (m)^2.

ASSESSMENT OF ISCHEMIC STROKE CASES

The outcome measure was incident ischemic stroke and ischemic stroke subtypes. Potential ischemic stroke cases were obtained by linkage to the Danish National Patient Register.[61] Participants registered with an ICD-8 discharge diagnose of 430, 431, 433, 434, 436.01, or 436.90 or an ICD-10 discharge code of I60, I61, I63, or I64 were considered potential stroke cases. The World Health Organization's definition of stroke as "an acute disturbance of focal or global cerebral function with symptoms lasting more than 24 hours or leading to death of presumed vascular origin" was used.[62] Case records were individually reviewed by a physician with neurological experience and diagnoses were validated and characterized based on clinical appearance, computed tomography, magnetic resonance imaging scan, autopsy records, spinal fluid examination, and other relevant information.[63] Ischemic stroke cases were subtyped according to the TOAST classification and included large artery atherosclerosis, cardioembolism, small-vessel occlusion, stroke of other etiology, and stroke of undetermined etiology.[3]

STATISTICAL ANALYSES

All statistical analyses were performed using Stata 14 (StataCorp LP). The endpoint in the four studies was total ischemic stroke and ischemic stroke subtypes according to the TOAST classification system. Hazard ratios (HR) with 95 % confidence intervals (CI) were calculated using sex-stratified Cox proportional hazard regression models allowing baseline hazards to differ between men and women. Attained age was the underlying time axis and observation time for each participant was the period from date of enrollment until occurrence of ischemic stroke, death from another cause, emigration, or end of follow-up (December 30th, 2009).

In the substitution analyses, exposures were investigated linearly as continuous variables. We included a sum-variable and each component separately except for the component to be replaced.[64] In the analyses of substitution of LA for other macronutrients the sum-variable was made from the sum of LA, SFA, MUFA, glycemic carbohydrate and protein in the diet. In the analyses of substitution of fish for other food-items the sum-variable was made from the sum of intake of fish, red meat and poultry. As the sum-variable held the total amount of components constant a higher intake from the component that was investigated, implied a concomitant lower intake from the component that was not included in the model. Thereby the difference in risk of ischemic stroke could be estimated for a 5% higher energy intake of LA and

a 5% lower energy intake of either SFA, MUFA or glycemic carbohydrates. Also, the risk of ischemic stroke could be estimated with a 150 g/week higher intake of fish and a concomitant lower intake of processed or unprocessed red meat or poultry.

Intake of total marine n-3 PUFA, EPA, DPA and DHA was investigated according to quartiles using the lowest quartile as the reference. Energy contribution from marine n-3 PUFA was too low to be investigated in a substitution model. Hence, analyses of dietary intake of total marine n-3 PUFA, EPA, DPA and DHA exposure were investigated using the residual method to energy-adjust these nutrients.[65] By using this approach, we were able to investigate dietary composition of total marine n-3 PUFA, EPA, DPA and DHA in relation to risk of ischemic stroke independent of total energy intake. This limits misclassification of intake of nutrients due to differences in physical activity, body size and metabolic efficiency.[65]

In the adipose tissue analyses exposures were investigated according to quartiles using the lowest quartile as the reference. We used a case-cohort design, which allowed us to limit costly gas chromatography analyses of adipose tissue to all cases and the subcohort. By performing weighted Cox regression analyses, HRs could be obtained as if the full cohort had been included. Participants were assigned weights, 1 for cases and N/n for noncases in the subcohort, where N was the number of noncases in the cohort and n was the number of noncases in the subcohort.[66] We carried out a Wald test for trend across quartiles.

In the analyses of ischemic stroke subtypes, only participants with a diagnosis of the ischemic stroke subtype in question were included as cases. Other subtypes of ischemic stroke were censored at the time of diagnosis since their risk of another stroke might have been changed.

Confounders were chosen a priori based on existing literature. We used three different models to adjust for potential confounding:

- Model 1A represented an age -and sex adjusted model. In the substitution analyses it also included total energy intake.
- Model 1B was a socioeconomic and lifestyle adjusted model. In model 1B, model 1A was further adjusted for: education, waist circumference adjusted for BMI, smoking, physical activity, alcohol intake and alcohol abstain.
- In Model 2 we further adjusted for other known risk factors for ischemic stroke, which also represented possible intermediate factors in the path between the exposures and ischemic stroke and included: hypertension, hypercholesterolemia, diabetes mellitus and atrial fibrillation/atrial flutter.

Restricted cubic splines with three knots were used to adjust for continuous variables. We evaluated the proportional hazards assumption in the Cox regression analyses by plotting scaled Schoenfeld residuals.

We used radar charts to investigate possible differences in the underlying dietary pattern in relation to intake or adipose tissue content of the macronutrient or food in question. Intake of different foods was energy adjusted using the residual method.

CHAPTER 5. STUDIES

This thesis is based on four studies conducted within the Diet, Cancer and Health cohort (**Figure 4**). The enrollment process was described in chapter 4.

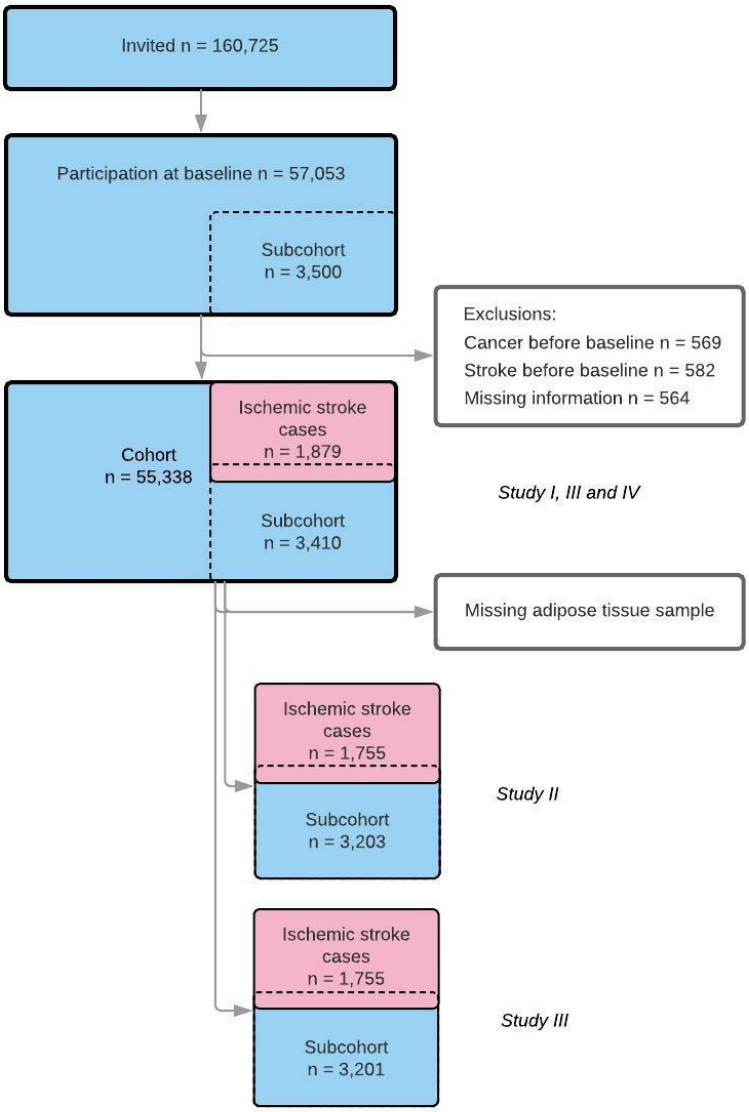

***Figure 4**: Flowchart of the study population used in the four studies.*

Of the 57,053 participants who agreed to participate, we excluded 569 participants with cancer and 582 participants with a diagnosis of stroke before enrollment. The information on covariates was insufficient in 564 participants, who were also excluded. Hence the final study population included 55,338 participants and the subcohort included 3,410 participants.

Participants were followed for a median of 13.5 years and during that time a total of 1879 ischemic strokes occurred. Baseline characteristics of the cohort, subcohort and ischemic stroke cases are shown in Table 1. When compared to participants in the cohort and in the subcohort, ischemic stroke cases were older, and a higher proportion was men. Also, ischemic stroke cases had a shorter education and a larger waist circumference, were more likely to be smokers, less physically active and had a higher alcohol intake. Furthermore, ischemic stroke cases were more likely to have hypercholesterolemia, hypertension, diabetes mellitus and atrial fibrillation/atrial flutter when compared to the cohort and subcohort.

***Table 2**. Baseline characteristics of the cohort, subcohort and ischemic stroke cases in the Diet, Cancer and Health cohort.*

	Cohort	Subcohort	Ischemic stroke cases
Age	56.1 (50.7-64.2)	56.3 (50.7-64.2)	58.9 (51.0-64.6)
Sex % (n)			
Male	47.6 (26,351)	54.1 (1,731)	61.9 (1,087)
Female	52.4 (28,987)	45.9 (1,470)	38.1 (668)
Education % (n)			
<7 years	32.9 (18,177)	32.9 (1,053)	41.0 (719)
8-10 years	46.1 (25,515)	45.0 (1,439)	42.6 (747)
>10 years	21.1 (11,646)	22.2 (709)	16.5 (289)
BMI (kg/m^2)	25.5 (20.5-33.3)	25.8 (20.7-33.4)	26.3 (21.0-34.9)
Waist circumference (cm)	88.8 (69.0-110.0)	90.0 (69.5-111.0)	93.0 (72.0-116.0)
Smoking status % (n)			
Non current	64.1 (35,462)	63.9 (2,045)	50.5 (887)
Current <15 g/d	13.0 (7,214)	13.6 (436)	15.4 (271)
Current ≥15 g/d	22.9 (12,662)	22.5 (720)	34.0 (597)
Physical activity (hours/week)	2.5 (0.0-11.0)	2.5 (0.0-10.5)	2.0 (0.0-11.0)
Alcohol intake (g/d)	12.9 (0.7-64.6)	13.8 (0.8-65.4)	14.5 (0.5-79.4)
Alcohol abstain % (n)			
Yes	2.3 (1,271)	2.1 (68)	3.0 (52)
No	97.7 (54,067)	97.9 (3,135)	97.0 (1,703)
Hypercholesterolemia % (n)			
Yes	7.4 (4,065)	7.9 (253)	10.8 (190)
No	50.3 (27,830)	49.3 (1,579)	48.4 (850)
Unknown	42.4 (23,443)	42.8 (1,369)	40.8 (715)
Hypertension % (n)			
Yes	16.0 (8,865)	15.7 (502)	28.8 (505)
No	70.9 (39,226)	71.7 (2,295)	57.7 (1,012)
Unknown	13.4 (7,147)	12.6 (404)	13.6 (238)
Diabetes mellitus % (n)			
Yes	2.0 (1,116)	2.0 (65)	4.3 (76)
No	93.4 (51,660)	92.9 (2,972)	89.6 (1,572)
Unknown	4.6 (2,562)	5.1 (164)	6.1 (107)
Atrial fibrillation/atrial flutter % (n)			
Yes	0.8 (54,915)	0.9 (30)	1.4 (24)
No	99.2 (423)	99.1 (3,171)	98.6 (1,731)

Values are expressed as medians (5^{th} -95^{th} percentile) and percent (number) for categorical variables. BMI indicates body mass index.

STUDY I

Aim

The aim of the first study was to investigate the association between a higher intake of LA and a concomitant lower intake of SFA, MUFA or glycemic carbohydrates in relation to ischemic stroke and its subtypes.

Key methods

Information on diet was assessed from the food frequency questionnaire that participants filled in at baseline. Statistical substitution models were used to investigate a 5% higher intake of LA and a concomitant lower intake of SFA, MUFA or glycemic carbohydrates. We compared participants with identical total energy intake and identical intake of macronutrients except for the macronutrient to be substituted. We used Cox proportional hazards regressions to estimate HRs with 95% CIs for ischemic stroke and its subtypes for these substitutions.

Main results

During follow-up, 1879 ischemic strokes occurred, including 319 cases of large artery atherosclerosis, 844 cases of small-vessel occlusion, 102 cases of cardioembolism, 98 strokes of other etiology, and 516 strokes of undetermined etiology.

A 5% higher intake of LA and a concomitant lower intake of SFA was associated with a slightly lower risk of total ischemic stroke and strokes caused by large artery atherosclerosis, although not statistically significant. A 5% higher intake of LA replacing MUFA was associated with a lower risk of total ischemic stroke and small-vessel occlusion, although only statistically significant for small-vessel occlusions. When a 5% higher intake of LA replaced glycemic carbohydrates a statistically non-significant lower risk of total ischemic stroke, strokes caused by large artery atherosclerosis and small-vessel occlusions were observed.

Main conclusions

Results from this study suggests that replacing SFA, MUFA or glycemic carbohydrate with LA may be associated with a lower risk of ischemic stroke.

Table 3. *Association between a 5 energy % higher intake of LA and a concomitant lower intake of SFA, MUFA or glycemic carbohydrates and risk of ischemic stroke and subtypes*

	LA for SFA	LA for MUFA	LA for glycemic carbohydrates
	HR (95% CI)	HR (95% CI)	HR (95% CI)
Total ischemic stroke	0.98 (0.83–1.16)	0.80 (0.63–1.02)	0.92 (0.78–1.09)
Large-artery atherosclerosis	0.84 (0.57–1.25)	1.05 (0.58–1.90)	0.96 (0.64–1.44)
Cardioembolism	1.46 (0.75–2.85)	1.35 (0.51–3.55)	1.55 (0.81–3.00)
Small-vessel occlusion	0.96 (0.75–1.23)	0.67 (0.46–0.96)	0.82 (0.64–1.05)
Stroke of other etiology	0.93 (0.45–1.91)	0.85 (0.29–2.45)	0.98 (0.47–2.05)

Adjusted for baseline age, sex, education, energy intake, waist circumference adjusted for BMI, smoking, physical activity, alcohol intake and alcohol abstain (Model 1B). LA indicates linoleic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; HR, hazard ratio and CI, confidence interval.

STUDY II

Aim

The aim of this study was to investigate the association between adipose tissue content of LA (an objective marker of intake and metabolism) and the risk of ischemic stroke and its subtypes.

Key methods

We conducted a case-cohort study nested within the Diet, Cancer and Health cohort. Ischemic stroke cases and a randomly drawn subcohort (n = 3500) had their adipose tissue biopsies analyzed by gas chromatography. Data were analyzed using weighted Cox proportional hazard regression.

Main results

Adipose tissue biopsies were available for 3203 participants in the subcohort and for 1755 ischemic stroke cases including 300 strokes caused by large artery atherosclerosis, 781 strokes caused by small-vessel occlusion, 99 strokes caused by cardioembolism, 91 strokes of other etiology, and 484 strokes of undetermined etiology.

Comparing the highest and the lowest quartiles of adipose tissue content of LA, we found a statistically significant inverse association with the rate of total ischemic stroke and large artery atherosclerosis. For small-vessel occlusion an inverse association with adipose tissue content of LA was found, although not statistically significant. There was no clear association between adipose tissue content of LA and the rate of cardioembolism.

Main conclusions

The content of LA in adipose tissue was statistically significantly inversely associated with the risk of total ischemic stroke and stroke caused by large artery atherosclerosis and statistically non-significantly inversely associated with small-vessel occlusion.

Table 4*. Adipose tissue content of LA in quartiles and association with ischemic stroke and ischemic stroke subtypes.*

	LA
	HR (95% CI)
Total ischemic stroke	
Q1	1
Q2	0.92 (0.77–1.09)
Q3	0.85 (0.71–1.02)
Q4	0.78 (0.65–0.93)
P_{trend}	P=0.004
Large artery atherosclerosis	
Q1	1
Q2	0.72 (0.51–1.01)
Q3	0.84 (0.61–1.17)
Q4	0.61 (0.43–0.88)
P_{trend}	P=0.021
Cardioembolism	
Q1	1
Q2	1.28 (0.75–2.19)
Q3	0.71 (0.37–1.37)
Q4	0.86 (0.46–1.59)
P_{trend}	P=0.311
Small-vessel occlusion	
Q1	1
Q2	0.90 (0.72–1.13)
Q3	0.87 (0.69–1.03)
Q4	0.87 (0.69–1.11)
$\mathbf{P_{trend}}$	P=0.236
Adjusted for baseline age, sex, education, waist circumference adjusted for body mass index, smoking, physical activity, alcohol intake and alcohol abstain (Model 1B). LA indicates linoleic acid; Q, quartile; HR, hazard ratio and CI, confidence interval.	

STUDY III

Aim

The aim of this study was to investigate the association between total marine n-3 PUFA, EPA and DHA from dietary intake and adipose tissue content in relation to ischemic stroke and its subtypes.

Key methods

Dietary intake of total marine n-3 PUFA, EPA and DHA was expressed as energy-adjusted intake in g/d. We used Cox proportional hazard regressions to analyze data of dietary intake. For the analyses of adipose tissue content of total marine n-3 PUFA, EPA and DHA we used a case-cohort design. Adipose tissue biopsies were analyzed using gas chromatography and a weighted Cox proportional hazard regression was used to analyze data.

Main results

The cohort included 55,338 participants for the analyses of dietary intake. During follow-up, 1879 participants developed ischemic stroke for whom 1755 adipose biopsies were available while 3201 participants had an adipose biopsy available within the subcohort. Ischemic stroke cases were distributed as given below with available adipose tissue biopsies in parentheses: 319 strokes due to large artery atherosclerosis (300), 102 strokes due to cardioembolism (99), 844 small-vessel occlusion strokes (781), 98 strokes of other etiology (91), and 516 strokes of undetermined etiology (484).

There was no association between intake or adipose tissue content of total marine n-3 PUFA and total ischemic stroke. However, adipose tissue content of EPA showed an inverse association with total ischemic stroke. Also, lower rates of large artery atherosclerosis were seen with higher intakes of total marine n-3 PUFA, EPA and DHA and higher adipose tissue content of EPA. Higher rates of cardioembolism were seen with higher intakes of total marine n-3 PUFA and DHA as well as with higher adipose tissue content of total marine n-3 PUFA and DHA. The EPA content in adipose tissue was inversely associated with small-vessel occlusion.

Main conclusions

Dietary intake and adipose tissue content of EPA was associated with a lower risk of most types of ischemic stroke, while total n-3 PUFA and DHA showed inconsistent results.

Table 5*. Dietary intake of total marine n-3 PUFA, EPA and DHA in quartiles and association with ischemic stroke and ischemic stroke subtypes.*

	Total marine n-3 PUFA	**EPA**	**DHA**
	HR (95% CI)	HR (95% CI)	HR (95% CI)
Total ischemic stroke			
Q1	1	1	1
Q2	1.06 (0.93-1.21)	1.05 (0.92-1.20)	1.08 (0.95-1.23)
Q3	1.06 (0.93-1.21)	1.09 (0.96-1.24)	1.02 (0.90-1.67)
Q4	1.06 (0.93-1.20)	1.01 (0.89-1.15)	1.06 (0.94-1.21)
P_{trend}	P = 0.458	P = 0.732	P = 0.513
Large artery atherosclerosis			
Q1	1	1	1
Q2	0.97 (0.72-1.30)	0.86 (0.64-1.16)	0.90 (0.67-1.22)
Q3	0.88 (0.65-1.19)	0.82 (0.60-1.11)	0.86 (0.63-1.16)
Q4	0.69 (0.50-0.95)	0.66 (0.48-0.91)	0.72 (0.53-0.99)
P_{trend}	P = 0.020	P = 0.012	P = 0.043
Cardioembolism			
Q1	1	1	1
Q2	1.36 (0.69-2.66)	1.17 (0.58-2.35)	0.97 (0.50-1.89)
Q3	1.49 (0.78-2.88)	2.34 (1.27-4.30)	1.27 (0.68-2.36)
Q4	2.50 (1.38-4.53)	2.02 (1.09-3.73)	2.12 (1.21-3.69)
P_{trend}	P = 0.001	P = 0.005	P = 0.002
Small-vessel occlusion			
Q1	1	1	1
Q2	1.15 (0.94-1.40)	1.14 (0.94-1.38)	1.26 (1.04-1.54)
Q3	1.20 (0.98-1.45)	1.16 (0.96-1.41)	1.17 (0.96-1.43)
Q4	1.06 (0.87-1.30)	1.05 (0.86-1.28)	1.13 (0.93-1.38)
P_{trend}	P = 0.518	P = 0.647	P = 0.411
Adjusted for baseline age, sex, education, waist circumference adjusted for body mass index, smoking, physical activity, alcohol intake and alcohol abstain (Model 1B). PUFA indicates polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosapentaenoic acid; Q, quintile; HR, hazard ratio and CI, confidence interval.			

***Table 6**. Adipose tissue content of total marine n-3 PUFA, EPA and DHA in quartiles and association with ischemic stroke and ischemic stroke subtypes.*

	Total marine n-3 PUFA	**EPA**	**DHA**
	HR (95% CI)	HR (95% CI)	HR (95% CI)
Total ischemic stroke			
Q1	1	1	1
Q2	0.98 (0.82-1.17)	0.91 (0.77-1.07)	0.92 (0.77-1.10)
Q3	1.12 (0.94-1.33)	0.66 (0.55-0.81)	1.09 (0.91-1.30)
Q4	1.08 (0.90-1.30)	0.74 (0.62-0.88)	1.00 (0.83-1.20)
P_{trend}	P = 0.213	P < 0.001	P = 0.580
Large artery atherosclerosis			
Q1	1	1	1
Q2	0.86 (0.61-1.22)	0.96 (0.70-1.32)	0.94 (0.67-1.32)
Q3	1.09 (0.78-1.52)	0.64 (0.43-0.94)	1.08 (0.77-1.52)
Q4	0.78 (0.53-1.13)	0.52 (0.36-0.76)	0.79 (0.54-1.16)
P_{trend}	P = 0.404	P < 0.001	P = 0.386
Cardioembolism			
Q1	1	1	1
Q2	2.08 (1.04-4.15)	1.13 (0.61-2.11)	1.37 (0.71-2.64)
Q3	2.04 (1.03-4.04)	1.06 (0.52-2.14)	1.64 (0.87-3.10)
Q4	2.63 (1.33-5.19)	1.52 (0.82-2.81)	2.00 (1.04-3.84)
P_{trend}	P = 0.007	P = 0.183	P = 0.030
Small-vessel occlusion			
Q1	1	1	1
Q2	0.91 (0.72-1.15)	0.84 (0.68-1.04)	0.86 (0.68-1.09)
Q3	1.05 (0.83-1.32)	0.61 (0.47-0.79)	1.07 (0.85-1.34)
Q4	0.99 (0.79-1.26)	0.69 (0.55-0.88)	0.92 (0.72-1.17)
P_{trend}	P = 0.768	P < 0.001	P = 0.916

Adjusted for baseline age, sex, education, waist circumference adjusted for body mass index, smoking, physical activity, alcohol intake and alcohol abstain (Model 1B). PUFA indicates polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosapentaenoic acid; Q, quintile; HR, hazard ratio and CI, confidence interval.

STUDY IV

Aim

The aim of this study was to investigate substitutions of one serving of fish per week for one serving of red meat or poultry in relation to ischemic stroke and its subtypes.

Key methods

Information on food-items was obtained from the food-frequency questionnaire. We used statistical food substitution models to investigate the rate of ischemic stroke and its subtypes when intake of 150 g/week of total, lean or fatty fish replaced processed or unprocessed red meat or poultry. Cox proportional hazard regression analyses were used to estimate associations between food substitutions and ischemic stroke risk.

Main results

A total of 1879 participants developed ischemic stroke, including 319 cases caused by large artery atherosclerosis, 844 small-vessel occlusions, 102 cases caused by cardioembolisms, 98 strokes of other etiology, and 516 strokes of undetermined etiology.

Total, lean or fatty fish replacing red meat or poultry was not associated with the rate of total ischemic stroke. However, a statistically significant lower rate of large artery atherosclerosis was found, when fish replaced processed and unprocessed red meat. When total fish replaced poultry a statistically significant higher rate of cardioembolism was found. A statistically significant lower rate of small-vessel occlusion was found when unprocessed red meat was replaced by fatty fish.

Main conclusions

In conclusion, replacement of red meat with fish was not associated with the risk of total ischemic stroke but was associated with a lower risk of large artery atherosclerosis.

***Table 7**. Substitution of total fish for processed red meat, unprocessed red meat or poultry and total ischemic stroke and ischemic stroke subtypes.*

	Total fish		
	Processed red meat	Unprocessed red meat	Poultry
	HR (95% CI)	HR (95% CI)	HR (95% CI)
Total ischemic stroke	0.97 (0.91-1.02)	0.97 (0.93-1.02)	1.00 (0.93-1.07)
Large artery atherosclerosis	0.78 (0.67-0.90)	0.87 (0.75-0.99)	0.83 (0.69-1.01)
Cardioembolism	1.26 (0.99-1.59)	1.14 (0.96-1.35)	1.42 (1.04-1.93)
Small-vessel occlusion	1.00 (0.92-1.10)	0.95 (0.88-1.02)	0.94 (0.85-1.04)
Adjusted for baseline age, sex, energy intake, education, waist circumference adjusted for body mass index, smoking, physical activity, alcohol intake and alcohol abstain (Model 1B). HR indicates hazard ratio and CI, confidence interval.			

***Table 8**. Substitution of lean fish for processed red meat, unprocessed red meat or poultry and total ischemic stroke and ischemic stroke subtypes.*

	Lean fish		
	Processed red meat	Unprocessed red meat	Poultry
	HR (95% CI)	HR (95% CI)	HR (95% CI)
Total ischemic stroke	0.98 (0.91-1.06)	0.99 (0.92-1.06)	1.01 (0.93-1.11)
Large artery atherosclerosis	0.77 (0.63-0.95)	0.86 (0.70-1.06)	0.83 (0.65-1.05)
Cardioembolism	1.28 (0.97-1.71)	1.16 (0.92-1.47)	1.45 (1.02-2.06)
Small-vessel occlusion	1.06 (0.95-1.19)	1.00 (0.91-1.11)	1.00 (0.88-1.13)
Adjusted for baseline age, sex, energy intake, education, waist circumference adjusted for body mass index, smoking, physical activity, alcohol intake and alcohol abstain (Model 1B). HR indicates hazard ratio and CI, confidence interval.			

***Table 9**. Substitution of fatty fish for processed red meat, unprocessed red meat or poultry and total ischemic stroke and ischemic stroke subtypes.*

	Fatty fish		
	Processed red meat	Unprocessed red meat	Poultry
	HR (95% CI)	HR (95% CI)	HR (95% CI)
Total ischemic stroke	0.95 (0.87-1.03)	0.95 (0.88-1.04)	0.98 (0.89-1.08)
Large artery atherosclerosis	0.78 (0.61-0.98)	0.87 (0.69-1.09)	0.84 (0.65-1.09)
Cardioembolism	1.22 (0.87-1.70)	1.10 (0.82-1.48)	1.37 (0.93-2.02)
Small-vessel occlusion	0.93 (0.81-1.07)	0.88 (0.77-0.99)	0.87 (0.75-1.01)
Adjusted for baseline age, sex, energy intake, education, waist circumference adjusted for body mass index, smoking, physical activity, alcohol intake and alcohol abstain (Model 1B). HR indicates hazard ratio and CI, confidence interval.			

CHAPTER 6. METHODOLOGICAL CONSIDERATIONS

In observational cohort studies, bias can occur due to problems with selection, information or confounding. Potential biases in the four studies in this thesis are discussed below.

SELECTION PROBLEMS

Selection bias may arise from systematic errors in procedures used to recruit participants into the study. Such errors may occur if the association between exposure and disease is different for participants and non-participants. An advantage of a prospective cohort study design is that the information on exposures is assessed at inclusion when participants are free from the disease of interest.

Selection bias can also be introduced if the investigated exposure gives rise to different completeness of follow-up. Ischemic stroke cases within the Diet, Cancer and Health cohort were identified through linkage to The Danish Patient Register, independently of the exposure in question. This made the follow-up almost complete, limiting the risk of selection bias.

In the Diet, Cancer and Health cohort, only 35% of those invited, agreed to participate and participants of a higher socioeconomic status were slightly overrepresented[56] but the investigated associations are believed not to differ across socio-economic groups. However, this is not selection bias but a problem with generalizability. Another generalizability issue is that participants in the Diet, Cancer and Health cohort were at recruitment living in and around Copenhagen and Aarhus in Denmark. Because participants were at least 50 years at inclusion, our results may only be applicable for people this age. Moreover, the study population was nearly exclusively Caucasians and findings may therefore not apply to other ethnic groups.

INFORMATION PROBLEMS

Information problems occur when measurements of exposure or outcome are not valid. Information problems may be introduced by the observer, by the

participants in the study or by the measurement instruments. For categorical variables, information problems are often referred to as misclassification.

Misclassification of exposure

Information on dietary intake in Study I, III and IV was assessed from self-reported food frequency questionnaires at baseline. Dietary food frequency questionnaires are known to be prone to measurement errors. However, the food frequency questionnaire used in this study, has been carefully developed and validated against two times seven days of weighted dietary records.[57,59] The food frequency questionnaires were designed to assess usual frequency of consumption during the last 12 months and therefore reflecting a relative long-term dietary intake. However, multiple measurements during follow-up of dietary intake would have captured potential dietary changes and would have been preferable to a single baseline measurement. Intake of fatty acids is generally problematic to assess through food frequency questionnaires due to difficulty in quantifying fat used in food preparation. Intake of LA is particularly difficult to assess because it is consumed from many different sources and can be hard to distinguish from other PUFA such as ALA.[42]

In Study II and III the exposure investigated was adipose tissue content of different PUFA. Adipose tissue provides an objective measure of fatty acid composition reflecting the endogenous exposure of individual fatty acids of the body.[67] Content of fatty acids in adipose tissue is influenced by consumption, uptake, synthesis, metabolism and release. LA cannot be synthetized in the body, while EPA and DHA can be synthetized from ALA, although to a limited extent. Therefore, adipose tissue content is a good biomarker of LA, EPA and DHA intake. Because of a slow turnover time, adipose tissue has been proposed to reflect dietary intake of these fatty acids during the previous 1-3 years.[67] However, adipose tissue biopsies were only obtained at baseline and potential changes in fatty acids in adipose tissue during follow-up were not assessed. Also, fatty acids in adipose tissue are measured as percentage of total fatty acids, and therefore influenced by the amount of other fatty acids. Food-frequency questionnaires and adipose tissue biopsies each have their advantages and disadvantages when used to examine long-term intake of LA, EPA and DHA. However, as complementary information they are useful tools.[68]

Misclassification of outcomes

The outcome in the four studies was ischemic stroke and its subtypes. Information on ischemic stroke cases was obtained by linkage with the Danish National Patient Register independently of the exposure in question. Thus, information bias is of no concern. Moreover, all cases of stroke were

individually validated.[63] Ischemic stroke cases were subtyped according to the TOAST classification system but a subset of 30 % with incomplete evaluations or two or more potential causes identified were categorized as stroke of undetermined etiology. Therefore, many ischemic stroke cases could not be classified into the three major etiological subtypes of strokes.

CONFOUNDING

A confounder is a factor that biases the association aimed to be studied, unless it is controlled for. It is associated with the exposure and independently affect the risk of the outcome. However, if a factor is an intermediate step in the causal pathway between the exposures of interest and the disease in question, it is not a confounder but a part of the effect being studied.[69,70]

Confounding is an important threat to the internal validity of observational studies. In studies of diet and diseases a confounder may introduce a false association between diet and disease. Importantly, participants who eat healthy are more likely to follow a healthy lifestyle e.g. by drinking less alcohol, not smoke and being physically active and if those variables are not controlled for it may distort the true relationship between the exposure being examined and the disease of interest. In the Diet, Cancer and Health cohort detailed information on social factors and lifestyle habits were collected at baseline from a self-reported lifestyle questionnaire. Also, anthropometric measurements were obtained at baseline. Thereby the participants provided information on potential confounders that we adjusted for, which limits but does not eliminate residual confounding.

Adjustment for potential confounders was applied in steps in different models. The first model represented an age and sex adjusted model (Model 1A). In the second model education and lifestyle factors were added to Model 1A and named Model 1B. For all analyses, when adding these covariates, the patterns of associations remained the same, although associations were weakened, indicating confounding. In Model 2 we added other known risk factors, hypercholesterolemia, hypertension, diabetes mellitus and atrial fibrillation/atrial flutter which may also be considered intermediate factors between the exposures and ischemic stroke. However, adjustment may introduce collider stratification bias and induce associations between factors affecting these variables. Thus, we considered Model 1B our main analyses with the most straightforward interpretation.

When ischemic strokes were subtyped according to the TOAST-classification system, the number of cases with cardioembolism and stroke of other etiology was low. This limited adjustment for potential confounders and risk factors.

CHAPTER 7. DISCUSSION

The aim of this thesis was to describe the association between n-6 and marine n-3 PUFA from dietary intake and content in adipose tissue and the risk of ischemic stroke and its subtypes. Dietary intake of LA replacing SFA, MUFA or glycemic carbohydrates was investigated in relation to ischemic stroke and its subtypes (Study I). Results indicated a lower risk of ischemic stroke and its subtypes with these substitutions. Also, adipose tissue content of LA was inversely related to ischemic stroke and its subtypes (Study II). We investigated both dietary intake and adipose tissue content of total marine n-3 PUFA, EPA and DHA in relation to ischemic stroke (Study III). The adipose tissue content of EPA was associated with a lower risk of most types of ischemic stroke while intake of total marine n-3 PUFA, EPA and DHA was associated with a lower risk of large artery atherosclerosis. Furthermore, consumption of fish replacing red meat or poultry was investigated in relation to ischemic stroke and its subtypes (Study IV). We found no association with total ischemic stroke with these substitutions but a lower risk of stroke due to large artery atherosclerosis was found when fish replaced red meat.

LINOLEIC ACID AND THE RISK OF ISCHEMIC STROKE

In Study I, a higher intake of LA and a concomitant lower intake of SFA, MUFA or glycemic carbohydrates was associated with a lower risk of ischemic stroke and its subtypes. These results were in agreement with our hypothesis. To date, this is to our knowledge the first study to investigate associations between substitution of LA for other macronutrients and ischemic stroke and its subtypes.

The association between dietary intake of n-6 PUFA (LA + AA) and ischemic stroke was investigated in The Swedish Mammography Cohort.[71] During 10.4 years of follow-up, 1310 participants developed ischemic stroke. No association was found between intake of n-6 PUFA and risk of ischemic stroke (HR: 0.96, 95% CI: 0.80-1.15, when comparing the highest and the lowest quintile). However, in this study substitutions were not specified.

While no other study has seemingly investigated substitution of LA for other macronutrients in relation to risk of ischemic stroke, the substitution aspect has been investigated in relation to another atherosclerotic disease, CHD. Hence, in a meta-analysis of 13 prospective cohort studies and a total of 310,602 participants Farvid et al.[72] found a lower risk of CHD when a 5% energy intake of LA replaced carbohydrates (HR: 0.90 95% CI, 0.85–0.94) or

SFA (HR: 0.91; 95% CI: 0.87–0.96). Similarly, another meta-analysis by Jakobsen et al.[73] of 11 prospective cohort studies and a total of 344,696 participants reported that a 5% higher intake of PUFA and a concomitant lower intake of SFA was associated with a lower risk of CHD (HR: 0.87; 95% CI: 0.77, 0.97).

Study II supports our a priori hypothesis that adipose tissue content of LA was inversely associated with risk of ischemic stroke and its subtypes. To our knowledge the association between adipose tissue content of LA has not been investigated previously, however, studies using serum content as a biomarker of LA has been investigated in relation to total ischemic stroke. Thus, in the Cardiovascular Health Study[74] the content of LA in plasma phospholipids was investigated in relation to ischemic stroke. In this prospective cohort study, 362 ischemic strokes occurred during 12.3 years of follow-up. Participants in the highest quintile of plasma phospholipid LA had a lower risk of ischemic stroke compared to participants in the lowest quintile, although not statistically significant (HR: 0.88; 95% CI: 0.61; 1.27). The association between plasma phospholipid LA was also investigated in the Kuopio Ischemic Heart Disease Risk Factor Study[75] and in this prospective cohort study, with a follow-up time of 21.2 years and 153 cases of ischemic stroke, no association between serum LA and ischemic stroke was found (HR: 1.07; 95% CI: 0.68-1.67, when comparing the highest and the lowest quartile of LA). Three nested case-control studies have investigated serum LA in relation to ischemic stroke. Yaemsiri et al.[76] included 964 ischemic stroke cases and found that serum LA was inversely associated with ischemic stroke, although not statistically significant. Iso et al.[77] included 122 ischemic stroke cases and found an inverse association between serum LA and ischemic stroke, which was statistically significant. However, DeGoede at al.[78] included 95 ischemic stroke cases and found no association between serum LA and risk of ischemic stroke. A possible mechanism for LA to reduce ischemic stroke risk is through beneficial effects on blood lipids. Thus, a meta-analysis of 60 trials showed that replacing carbohydrates with polyunsaturated fat (mainly LA) was associated with a lower plasma LDL cholesterol.[52] Other possible biologic mechanisms by LA could be a lowering of blood pressure[79] or an improvement of insulin sensitivity.[80]

Evidence from the radar chart in or Study II, implied that a high adipose tissue content of LA was associated with overall healthier eating habits and results can therefore not be directly interpreted as associations for LA independently of eating patterns associated with LA intake. Combing evidence from our study with other published results, however, suggests a beneficial association of LA consumption, or a healthy diet including LA, on risk of ischemic stroke.

TOTAL MARINE N-3 PUFA, EPA AND DHA AND THE RISK OF ISCHEMIC STROKE

In Study III, we investigated the association of dietary intake of total marine n-3 PUFA, EPA and DHA in relation to ischemic stroke. Neither intake of total marine n-3 PUFA, EPA nor DHA was associated with the risk of ischemic stroke. However, intake of total marine n-3 PUFA, EPA and DHA was inversely associated with the risk of stroke due to large artery atherosclerosis. To our knowledge, no other studies have evaluated intake of total marine n-3 PUFA, EPA and DHA in relation to ischemic stroke subtypes. However, six other prospective cohort studies have investigated the association between intake of EPA + DHA and total ischemic stroke.

In the Cohort of Swedish Men,[81] intake of EPA + DHA was investigated in relation to ischemic stroke. In this prospective cohort study, including 2286 ischemic stroke cases, no association was found between intake of EPA + DHA and risk of ischemic stroke (HR: 1.09; 95% CI: 0.94–1.26, when comparing the highest and the lowest quartile) after 12 years of follow-up. The association between intake of EPA + DHA and ischemic stroke was also investigated in the Swedish Mammography Cohort.[71] In this prospective cohort study, women were followed for a mean of 10.4 years and 1,310 developed ischemic stroke. Intake of EPA + DHA was inversely associated with ischemic stroke risk, and when comparing the highest with the lowest quintile a HR of 0.83 (95% CI: 0.69–0.99) was found. The Health Professional Follow-up Study[82] followed men for 12 years and 377 developed an ischemic stroke. There was no dose-response association between intake of EPA + DHA and ischemic stroke. However, lower risks of ischemic stroke were found in each quintile except for the highest quintile compared to the lowest one (HR: 0.73; 95% CI: 0.43-1.25). In the Finnish Mobile Clinic Health Examination Survey,[83] with 28 years of follow-up and 364 ischemic stroke cases no association was found between intake of EPA + DHA and ischemic stroke (HR: 0.91; 95% CI: 0.66-1.26 when comparing the highest with the lowest quartile of intake). In contrast, a lower risk of ischemic stroke was found among women in the highest quintile compared to the lowest quintile of EPA + DHA intake (RR: 0.73; 95% CI: 0.43-1.25), although not statistically significant in the Nurses' Health Study.[77] Finally, in the Monitoring Project on Risk Factors for Chronic Diseases (Morgen) study,[84] inverse but not statistically significant associations were found between intake of EPA + DHA and risk of ischemic stroke for men (HR: 0.62; 95% CI: 0.29–1.35, comparing the highest with the lowest quartile of intake) and women (HR: 0.85; 95% CI: 0.45–1.60, comparing the highest with the lowest quartile of intake).

None of the studies mentioned above studied EPA and DHA separately in relation to ischemic stroke. However, EPA and DHA are consumed from the

same dietary sources and supposed to be highly correlated. In contrast, adipose tissue content of EPA and DHA offers an objective marker of individual fatty acids and is considered the gold standard according to a slow turnover time and therefore a good measure of long-time body availability of marine n-3 PUFA.[67]

In Study III, we also investigated adipose tissue content of total marine n-3 PUFA, EPA and DHA in relation to risk of ischemic stroke. Total marine n-3 PUFA and DHA showed no consistent association with ischemic stroke or its subtypes, but adipose tissue content of EPA was inversely associated with total ischemic stroke, stroke due to large artery atherosclerosis and small-vessel occlusions. To our knowledge, this is the only study reported to investigate adipose tissue content of total marine n-3 PUFA, EPA and DHA in relation to risk of ischemic stroke and its subtypes. However, short-term biomarkers of EPA, DPA and DHA have been investigated in relation to ischemic stroke and its subtypes in three other studies. Thus, a recent prospective study[85] investigated the content of EPA and DHA in plasma phospholipid in relation to ischemic stroke using pooled data from 3 cohorts: Cardiovascular Health Study, Nurses' Health Study and Health Professional Follow-Up Study, with a total of 953 ischemic stroke cases identified. When comparing the highest with the lowest quartile of DHA it was associated with a lower risk of total ischemic stroke and atherothrombotic stroke (large artery atherosclerosis + small-vessel occlusion) while there was no association between EPA and total ischemic stroke or atherothrombotic stroke. These findings opposed our findings of inverse associations between EPA, but not DHA, and ischemic stroke and its subtypes. Moreover, in a nested case-control study including 964 ischemic stroke cases, Yaemsiri et al.[76] found that serum DHA but not EPA was inversely associated with risk of total ischemic stroke and ischemic stroke subtypes. Finally, in another nested case-control study including 144 ischemic stroke cases, Iso et al.[86] reported no association between serum levels of total marine n-3 PUFA, EPA or DHA and the risk of ischemic stroke or its subtypes.

Three other studies have investigated biomarkers of marine n-3 PUFA in relation to total ischemic stroke risk. In The Cardiovascular Health Study[87] which is a prospective cohort study of 319 ischemic stroke cases, no association was found between plasma phospholipid EPA (HR: 1.09; 95% CI: 0.76–1.57) and risk of ischemic stroke. However, when comparing the highest and the lowest quartile of plasma phospholipid lower risks of ischemic stroke were found for DHA (HR: 0.74; 95% CI: 0.50–1.10) and total marine n-3 PUFA (HR: 0.63; 95% CI: 0.43–0.94). In the Kuopio Ischemic Heart Disease Risk Factor Study, which included 153 ischemic stroke cases, no association was found between serum EPA (HR: 1.27; 95% CI: 0.80-2.00), DHA (HR: 1.01; 95% CI: 0.70-1.69) or total marine n-3 PUFA (HR: 0.98; 95% CI: 0.64-1.51)

and ischemic stroke risk, when comparing the highest and the lowest quartile. Finally, in a case-control study nested in the prospective MORGAN study 93 ischemic stroke cases were identified with no association was found between the sum of EPA and DHA from plasma cholesteryl esters and ischemic stroke risk.

The mechanisms by which marine n-3 PUFA may reduce the risk of ischemic stroke include beneficial effects on blood pressure, plasma triglycerides, and platelet aggregability and inflammation.[38] In general, other studies have reported an association between biomarkers of DHA but not EPA on ischemic stroke risk. In contrast, in our study, adipose tissue content of EPA, but not DHA, was inversely associated with total ischemic stroke, large artery atherosclerosis and small-vessel occlusion, suggesting a protective effect of EPA against atherosclerotic disorders. Two randomized, placebo-controlled trials have investigated supplementation with highly purified EPA. In a sub-study of the Japan Eicosapentaenoic acid Lipid Intervention Study (Jelis),[88] supplementation with 1.8 g of purified EPA in hypercholesterolemic subjects a lower risk of cerebral thrombosis was found in a secondary prevention subgroup, although not statistically significant (HR: 0.72; 95% CI 0.50–1.00). Recently, the Reduce-it trial[89] investigated the effect of a supplement with 4 g of EPA in 8179 patients with established cardiovascular disease or risk factors and found a lower risk of fatal and nonfatal stroke among participants receiving EPA compared to placebo (HR: 0.72; 95% CI: 0.55-0.93). However, the study reported results only on total stroke without separating ischemic and hemorrhagic events.

Both EPA and DHA have been shown to lower serum triglyceride levels, however DHA has also been found in some studies to raise plasma LDL cholesterol levels.[89–92] LDL cholesterol is a key component of atherosclerosis, thus our different results of ischemic stroke subtypes seen among adipose tissue content of EPA and DHA might be related to effects on plasma LDL cholesterol levels.

In Study IV, dietary intake of fish replacing red meat or poultry was investigated in relation to risk of ischemic stroke and its subtypes. One portion of fish per week (corresponding to 150 g) replaced a portion of red meat or poultry. We found no association with total ischemic stroke with these substitutions but a lower risk of stroke due to large artery atherosclerosis when fish replaced red meat. Also, when unprocessed red meat and poultry was replaced by fatty fish lower risks of small-vessel occlusions were seen.

Several studies have evaluated intake of fish in relation to risk of ischemic stroke and some but not all found an inverse association.[93–95] However, most studies have adjusted for total energy intake in the analyses and when total

energy intake is included in the analyses an isoenergetic substitution model is created and a higher intake of the investigated food or macronutrient implies a lower intake of all other foods. Hence a substitution model is created, although rarely specified and the replacement food should be defined in the models. In the Nurses' Health Study, which is a prospective cohort study substitution of one serving a day of fish for red meat or poultry was also investigated.[96] A total of 4,030 strokes were identified and lower risks of strokes were found when fish replaced red meat (RR: 0.83; 95% CI: 0.70-1.00) and when poultry replaced fish (RR: 0.89; 95% CI: 0.68-1.16). However, substitutions of food groups were only investigated in relation to total stroke hence results are not directly comparable to our results.

In conclusion, combining our results with results from other studies may imply a beneficial effect of fish and marine n-3 PUFA intake on ischemic stroke risk, in particular strokes caused by large artery atherosclerosis although the associations between EPA and DHA and risk of ischemic stroke has differed between studies. Furthermore, results from our study suggest that the content of EPA in adipose tissue is inversely associated with ischemic stroke and its subtypes.

Ischemic stroke is a heterogenous disorder with mixed etiology and we hypothesized that associations of n-6 and marine n-3 PUFA may differ among subtypes of ischemic stroke. The TOAST classification system[3] offers a method for subtyping ischemic stroke according to etiology. Adipose tissue LA and EPA were inversely associated with strokes due to large artery atherosclerosis. Lower risks of large artery atherosclerosis were also seen with high intakes of total and individual marine n-3 PUFA as well as when SFA was replaced with LA and when red meat or poultry was replaced with fish.

Strokes due to small-vessel occlusion was the largest subgroup of ischemic stroke comprising more than 800 cases. Adipose tissue content of EPA was inversely associated with small-vessel occlusion. Also, when LA replaced MUFA or glycemic carbohydrates and when fatty fish replaced unprocessed red meat or poultry a lower risk of small-vessel occlusion was found. The biological mechanisms between PUFA and small-vessel occlusion are not entirely clear. Though there is an atherosclerotic component in the development small-vessel occlusion, one of the most important risk factors is hypertension. It is therefore of potential significance that intake of fish and marine n-3 PUFA are known to slightly lower blood pressure.[97]

We found no association between neither dietary substitution nor adipose tissue content of LA and strokes due to cardioembolism in Study I and II. However, a statistically significant higher risk of cardioembolism was found with a high intake and high adipose tissue content of marine n-3 PUFA in

Study III. Also, results from Study IV indicated higher risks of cardioembolism, when fish replaced red meat or poultry. The reason for this higher risk of cardioembolism is unclear. One possibility is that the most important source of cardioembolism is atrial fibrillation and some studies have reported a positive association between a high intake of fish as well as marine n-3 PUFA and risk of atrial fibrillation.[98–101] Also, results from the Reduce-it study[89] showed a statistically significant higher risk of atrial fibrillation/atrial flutter among participants receiving EPA compared to controls (3.1% vs. 2.1%, P=0.004). However, the number of cases with cardioembolism in our study was low and those results should be interpreted with caution.

Our results of the four studies suggest that the associations between n-6 and marine n-3 PUFA and ischemic stroke may not be consistent across subtypes of ischemic stroke underlining the need to investigate PUFA in relation to the individual ischemic stroke subtypes.

CHAPTER 8. CONCLUSIONS AND PERSPECTIVES

Our results from the four studies evaluating both dietary intake and content of PUFA in adipose tissue suggest:

- Substitution of dietary LA for SFA, MUFA or glycemic carbohydrates may be associated with a lower risk of ischemic stroke and its subtypes, apart from stroke due to cardioembolism.
- Adipose tissue content of LA may be associated with a lower risk of ischemic stroke and its subtypes, except for strokes due to cardioembolism.
- Intake of total marine n-3 PUFA, EPA and DHA may be associated with a lower risk of large artery atherosclerosis.
- Adipose tissue content of EPA may be associated with a lower risk of total ischemic stroke, large artery atherosclerosis and small-vessel occlusion.
- Adipose tissue content of total marine n-3 PUFA and DHA showed inconsistent associations with total ischemic stroke and its subtypes except for stroke due to cardioembolism for which a positive association was observed.
- Total and lean fish replacing processed, and unprocessed red meat may be associated with a lower risk of large artery atherosclerosis, while replacing poultry with total and lean fish may be associated with a higher risk of cardioembolism.
- Fatty fish replacing processed and unprocessed red meat and poultry may be associated with a lower risk of large artery atherosclerosis and small-vessel occlusion.

The association between substitution of LA and risk of ischemic stroke needs further investigation by other prospective studies in order to confirm the lower risk of ischemic stroke by increasing the intake of LA at expense of other macronutrients found in our study. Although adipose tissue content of LA showed a statistically significantly inverse association with the risk of ischemic stroke, this result should not be considered definitive and other large prospective cohort studies as well as randomized controlled studies are needed to confirm or reject our findings.

Both dietary intake and adipose tissue content of EPA and DHA in relation to ischemic stroke deserve further attention. Large prospective cohort studies and randomized controlled studies are necessary to confirm or reject the

observed inverse association between adipose tissue content of EPA and ischemic stroke and its subtypes. Also, experimental studies are needed to clarify the biologic mechanisms between EPA and DHA intake and etiology of ischemic stroke.

Evidence from this study suggests that long-term intake of different macronutrients is often associated with specific food patterns. Therefore, macronutrients should not be investigated isolated and future research should also target food patterns. There is especially a need for studies of substitutions in relation to ischemic stroke, which is applicable for both macronutrients and food items. Furthermore, results from substitution analyses of food items are easily translated into dietary guidelines.

Ischemic stroke is a heterogenous condition and our study results highlight the importance of investigating n-6 PUFA, marine n-3 PUFA and fish in relation to ischemic stroke subtypes separately. It would be desirable to pool data from other prospective cohort studies to increase number of cases for the analyses of subtypes of ischemic stroke. In contrary to our hypothesis, intake of marine n-3 PUFA was associated with a higher risk of cardioembolism. Future directions of research include prospective cohort studies with a larger number of cases of stroke due to cardioembolism in order to elucidate a potential association.

Taken together our findings suggest that an increased consumption of foods rich in n-6 and marine n-3 PUFA may lower the risk of ischemic stroke especially ischemic stroke of atherosclerotic origin, which supports recent guidelines.[50] Such a diet have also been associated with a lower risk of acute coronary syndrome[102,103] myocardial infarction[104–106] and peripheral arterial disease.[107,108] Furthermore, the study suggests, that associations depend on the macronutrients or food item to be replaced and most likely also on the dietary pattern that follows an increased intake of n-6 PUFA and marine n-3 PUFA.

CHAPTER 9. ENGLISH SUMMARY

Ischemic stroke is a leading cause of death and long-term disability worldwide. Though, n-6 and marine n-3 PUFA from the diet have been hypothesized to reduce ischemic stroke risk, previous studies have shown inconsistent results. The aim of this thesis was to investigate the association between marine n-3 and n-6 PUFA from dietary intake and content in adipose tissue and the risk of ischemic stroke and its subtypes.

For this thesis, we used the Diet, Cancer and Health cohort which included 57,053 Danish participants age 50-64 at baseline. All participants filled in a food-frequency questionnaire at baseline which was used to investigate dietary intake. Furthermore, participants had an adipose tissue biopsy taken at baseline and content of fatty acids in adipose tissue (a long-term indicator of diet and biological exposure) was analyzed by gas chromatography. Potential ischemic stroke cases were found by linkage to the Danish National Patient Register. All cases were individually validated and subtyped according to the TOAST-classification system.

During a median follow-up time of 13.5 year 1,879 ischemic strokes occurred. In Study I, the association between intake of LA and risk of ischemic stroke was investigated by use of substitution models. Hence, a 5% higher intake of LA and a concomitant lower intake of SFA, MUFA or glycemic carbohydrates was investigated. Results indicated a lower risk of ischemic stroke and ischemic stroke subtypes with these substitutions. In Study II, associations between adipose tissue content of LA to ischemic stroke and its subtypes was investigated and results indicated inverse associations. In Study III, association between both dietary intake and adipose tissue content of total marine n-3 PUFA, EPA and DHA and risk of ischemic stroke were studied. The adipose tissue content of EPA was associated with a lower risk of most types of ischemic stroke, while intake of total marine n-3 PUFA, EPA and DHA was associated with a lower risk of strokes due to large artery atherosclerosis. In Study IV, association between fish intake and ischemic stroke risk was investigated using substitution models where 150 g/week (corresponding to a portion size) of fish replaced 150 g/week of red meat or poultry. These substitutions were not associated with risk of total ischemic stroke however, a lower risk of large artery atherosclerosis strokes was observed when fish replaced red meat.

Overall, findings of this thesis indicate that increased intake of foods rich in n-6 PUFA and marine n-3 PUFA may be of benefit for primary prevention of ischemic stroke especially strokes of atherosclerotic origin.

CHAPTER 10. DANSK RESUME

Iskæmisk apopleksi er en hyppig årsag til død og invaliditet på verdensplan. Selvom n-6 og marine n-3 fedtsyrer i kosten menes at have en gavnlig effekt på risikoen for udvikling af iskæmisk apopleksi, har resultater fra tidligere studier været inkonsistente. Målet med denne afhandling var at undersøge sammenhængen mellem n-6 og marine n-3 fedtsyrer i kosten og indholdet i fedtvæv og risikoen for iskæmisk apopleksi samt undertyper heraf.

Denne afhandling er baseret på data fra den danske befolkningsundersøgelse Kost, kræft og helbred, som inkluderede 57,053 danske deltagere på 50-64 år ved rekrutteringstidspunktet. Alle deltagere udfyldte et fødevarefrekvens-spørgeskema ved studiestart, som blev brugt til at undersøge deltagernes vanlige kost. Derudover fik deltagerne ved studiestart taget en fedvævsbiopsi, som blev analyseret med gaskromatografi. Potentielle tilfælde af iskæmisk apopleksi blev fundet ved kobling med Landspatientregistret. Alle cases blev valideret og underopdelt på baggrund af TOAST-klassifikationssystemet.

I løbet af en median opfølgningstid på 13,5 år udviklede 1879 deltagere iskæmisk apopleksi. I Studie I blev associationen mellem indtag af linolsyre og risikoen for iskæmisk apopleksi undersøgt ved hjælp af substitutionsmodeller, hvorved et øget indtag af linolsyre og et tilsvarende mindre indtag af mættet fedt, monoumættet fedt eller glykæmiske kulhydrater blev undersøgt. Resultaterne viste en lavere risiko for iskæmisk apopleksi samt undertyper af iskæmisk apopleksi ved disse substitutioner. I Studie II blev associationen mellem linolsyre i fedtvæv og iskæmisk apopleksi samt undertyper undersøgt og resultaterne viste inverse associationer. I Studie III blev både indtaget samt koncentrationen af total marine n-3 fedtsyrer i fedtvæv og de to undertyper EPA og DHA undersøgt i forhold til risiko for iskæmisk apopleksi. Et højt indhold af EPA i fedtvæv var forbundet med en lavere risiko for de fleste typer af iskæmisk apopleksi imens indtag af både total marine n-3 fedtsyrer, EPA og DHA var forbundet med en lavere risiko for storkarsinfarkter. I Studie IV blev sammenhæng mellem indtag af fisk og risiko for udvikling af iskæmisk apopleksi undersøgt med substitutionsmodeller, idet 150 g fisk pr. uge (tilsvarende en normal portionsstørrelse) erstattede 150 g rødt kød eller fjerkræ pr. uge. Disse substitutioner var ikke associeret med risiko for udvikling af total iskæmisk apopleksi, imidlertid blev en lavere risiko for storkarsinfarkter observeret, når fisk blev udbyttet med rødt kød.

Samlet set indikerer resultaterne i denne afhandling, at et øget indtag af fødevarer rige på n-6 og marine n-3 fedtsyrer kan være fordelagtigt i forhold til primær forebyggelse af iskæmisk apopleksi, især apopleksi af aterosklerotisk oprindelse.

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APPENDICES

Appendix A. Paper I

Appendix B. Paper II

Appendix C. Paper III

Appendix D. Paper IV

Appendix A. Paper I

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Appendix B. Paper II

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Appendix C. Paper III

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Appendix D. Paper IV

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