Marine n-3 fatty acids and genetic variants in the 5-lipoxygenase pathway

association with myocardial infarction

Gammelmark, Anders

DOI (link to publication from Publisher):
10.5278/VBN.PHD.MED.00073

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

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ASSOCIATION WITH MYOCARDIAL INFARCTION

BY
ANDERS GAMMELMARK

DISSERTATION SUBMITTED 2016

AALBORG UNIVERSITY
DENMARK

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PhD thesis, submitted 2016

Dissertation submitted: August 23, 2016

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PhD Series: Faculty of Medicine, Aalborg University

ISSN (online): 2246-1302
ISBN (online): 978-87-7112-785-0

Published by:
Aalborg University Press
Skjernvej 4A, 2nd floor
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk

Printed in Denmark by Rosendahls, 2016

ACKNOWLEDGEMENTS

During my time as a PhD-student I was employed at the Department of Cardiology, Aalborg University Hospital, and spent most of my time at the Science and Innovation Center, Aalborg University Hospital. I had the privilege and opportunity to get to know many excellent colleagues and friends, many of whom have been a significant part of my life and contributed in different ways to the work of my PhD. Altogether, you have made this part of my life a great experience and I hope for many more to come in the future. I would like to pay a special thanks to a number of people.

First of all, to my supervisors, who have supported me unconditionally throughout the process:

Erik Berg Schmidt. We have known each other for quite some time and your guidance in many aspects of life and work are most appreciated. Thank you for always being there to consult and encourage me. I am most grateful for the many talks we have had along the way on many subjects and your example as a leader and scientist has been an inspiration. When times have been trying, you have always cared and known when to ask how I was doing, and when problems have seemed too hard to overcome you have always guided me towards the solution. Your dedication and highly qualified opinions are always valued.

Kim Overvad. Having you as a supervisor has been a great pleasure and your exceptional knowledge and experience in epidemiology has made a real difference for this PhD-project and for me. Along the way, your continuous guidance and integrity as a scientist and supervisor has been a huge inspiration, and your overview and sharp analyses has been comforting and reassuring to me.

Michael Skjelbo Nielsen. From the beginning of my period as a PhD-student, your contribution has been significant, and the conception of this PhD-project was inspired by the work from your own PhD-thesis. Your detailed knowledge on fatty acid metabolism and genetics has been a great help and your encouragement throughout my PhD have been exceptional. I hope that our relation will continue for many years to come.

Søren Lundbye-Christensen. As my statistical supervisor, your contribution and tutoring has been absolutely indispensable. I will always remember you for the many afternoons we spent discussing many subjects, some of them including some pretty complex genetical statistics. Your humorous and illustrative approach has been an inspiration and have made some quite daunting statistics quite fun.

I would also like to thank the staff at the Lipid Clinic for the time we have spent together, especially the morning coffee breaks, often including cake, or the afternoon coffee breaks, also often including cake along many friendly talks. To the staff at the Lipid lab, I have been delighted about your technical support and effort in providing me with adipose tissue analyses as fast as possible. To the study nurses, I am happy to have had the chance to get to know and work with you all, alongside my PhD-project. Thank you for your interest in both my PhD-work and my personal wellbeing. Last, but not the least, I owe a special thanks to Hanne Madsen for careful proofreading of both manuscripts and thesis, as well as help with many practical issues.

To my fellow PhD-students and Research year students that I encountered along my time at the Science and Innovation Center, Stine Venøe, Pia Dinesen, Thure Overvad, Vibeke Høgh, Jacob Mosgaard, Henrik Vadmann, Anne Lasota, Line Melgaard, Anette Højen, Thomas Rix, Jakob Stegger, Steen Hansen, Christina Graversen and Kasper Emmerik. Thank you all for contributing a friendly atmosphere, and a very inspiring scientific environment, and at times, very unscientific. I owe a special thanks to Christian Bork for assisting me in the validation of new cases and for his contribution to paper I and II.

For the studies included in this thesis I received financial support from The Danish Heart Association, Hertha Christensens Foundation, Special Doctor Heinrich Kopp's Grant and Reservelægefonden at Aalborg University Hospital, for which I am most grateful. However, the genotyping of the study cohort could not have been accomplished, had it not been for the Department of Cardiology, Aalborg University Hospital, who provided financial support when no other funding was available. I am enormously thankful for that. Finally, I feel privileged to have had the opportunity to work with data from the Diet, Cancer and Health Cohort, funded by the Danish Cancer Society, and I have enjoyed much practical as well as scientifical aid from the staff at the Danish Cancer Society.

Lastly, I would like to thank my family and friends for their continuous support and interest in my work. I owe a special gratitude to my wife, Carina, for her understanding and loving support, especially during the last year when working hours have been long and my contribution to the daily tasks has been sparse. To our three girls, Gry, Selma and Liv, thank you for keeping my spirits high and always putting a smile on my face. I hope to spend more time with you in the near future.

Anders Gammelmark, MD

August, 2016

ABBREVIATIONS

AA.....Arachidonic acid
ALA.....Alpha linolenic acid
ALOX-5.....Arachidonate 5-lipoxygenase
CDR.....The Danish causes of death registry
CHD.....Coronary heart disease
CVD.....Cardiovascular disease
DHA.....Docosahexaenoic acid
DPA.....Docosapentaenoic acid
EPA.....Eicosapentaenoic acid
FLAP.....5-lipoxygenase activating protein
ICD.....International classification of disease
5-LOX.....5-lipoxygenase
LA.....Linoleic acid
LDL.....Low-density lipoprotein
LTA_4.....Leukotriene A_4
LTA_4-H.....Leukotriene A_4 hydrolase
LTB_4.....Leukotriene B_4
LTB_5.....Leukotriene B_5
LTC_4-S.....Leukotriene C_4 synthase
MI.....Myocardial infarction
NPR.....The Danish national patient registry
PUFA.....Polyunsaturated fatty acids
RERI.....Relative excess risk due to interaction
SNP.....Single nucleotide polymorphism

LIST OF PAPERS

This thesis is based on the following papers:

Paper I
A. Gammelmark, M.S. Nielsen, C.S. Bork, S. Lundbye-Christensen, A. Tjønneland, K. Overvad, E.B. Schmidt. Fish consumption and dietary intake of marine n-3 PUFA are inversely associated with myocardial infarction in a prospective Danish cohort study. Br. J Nutr, 2016, 116, p167-177.

Paper II
A. Gammelmark, M.S. Nielsen, C.S. Bork, S. Lundbye-Christensen, A. Tjønneland, K. Overvad, E.B. Schmidt. Adipose tissue content of marine n-3 polyunsaturated fatty acids is inversely associated with myocardial infarction: A Danish case-cohort study. JACC, 2016, 67, p1008-1009.

Paper III
A. Gammelmark, M.S. Nielsen, S. Lundbye-Christensen, A. Tjønneland, E.B. Schmidt, K. Overvad. Common Polymorphisms in the 5-Lipoxygenase Pathway and Risk of Incident Myocardial Infarction: A Danish Case-Cohort Study. PLOS ONE, revision submitted, 18. August.

Paper IV
A. Gammelmark, S. Lundbye-Christensen, A. Tjønneland, E.B. Schmidt, K. Overvad, M.S. Nielsen. Diet-gene interaction between 5-lipoxygenase polymorphisms and substrates for the 5-lipoxygenase pathway modulates the risk of myocardial infarction - a Danish case-cohort study. Manuscript in draft.

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CHAPTER 1. INTRODUCTION

Cardiovascular disease (CVD) remains a leading cause of death in the industrialised world, causing about 30% of deaths globally according to the WHO (1). Despite great advances in the treatment of acute coronary events and the steady decline in the relative proportion of fatal coronary events, the burden of CVD is growing, and the prevalence of cardiac risk factors such as obesity, diabetes mellitus, hypertension, dyslipidaemia and other lifestyle factors associated with CVD risk continues to increase worldwide.

Atherosclerosis is the main pathophysiological component of CVD, and over the last two decades, our understanding of atherosclerosis has evolved. Today, atherosclerosis is indeed considered a multifactorial disorder, where inflammatory processes play a crucial role in the development and stability of atherosclerotic plaques preceding clinical symptoms of CVD.

There are many aspects of inflammation, including the leukotrienes, that have been associated with atherosclerosis traits. Leukotrienes belong to a class of highly pro-inflammatory lipid mediators that are synthesised through the 5-lipoxygenase (5-LOX) pathway, mainly derived from arachidonic acid (AA) and, to a more limited extent, eicosapentaenoic acid (EPA) – specific n-6 and n-3 polyunsaturated fatty acids (PUFA), respectively. Different types of PUFA give rise to different leukotrienes, and the 5-series leukotrienes derived from EPA are considerably less pro-inflammatory compared with 4-series leukotrienes derived from AA.

Consumption of marine n-3 PUFA found in seafood has been associated with a lower risk of coronary heart disease (CHD) in a number of observational studies and clinical intervention trials, and in contrast, some studies have associated AA with a higher risk of CHD. Thus, it has been proposed that consumption of n-3 PUFA may cause a shift in cell membrane lipids towards a higher content of EPA at the expense of AA, which may lead to the formation of less pro-inflammatory leukotrienes from EPA compared with AA.

Further, adding to the biological complexity, genetic variations in genes coding for enzymes involved in the leukotriene biosynthesis have been associated with risk of atherosclerotic disease, and it is plausible that subjects with genetic variants may up- or down-regulate leukotriene formation which may in turn interact with the dietary intake of n-3 and n-6 PUFA. Thus, evidence suggests a complex interplay between genetic variants related to the leukotriene pathway and dietary intake of AA and EPA, and it is plausible that dietary PUFA may influence the development and risk of CHD, at least partly, through their formation of different leukotrienes.

This PhD thesis is based on four studies from the Danish cohort study Diet, Cancer and Health. The cohort included a total of 57,053 participants and during follow-up we identified 3,089 cases of incident MI. A sub-cohort of 3,500 participants was randomly selected to represent the cohort in study II-IV. First, we investigated the association of dietary fish consumption and incident MI, and next, the association between the content of marine n-3 PUFA in adipose tissue and incident MI. By these complementary methods, we evaluated both a subjective measure of fish consumption and an objective measure of the endogenous exposure to marine n-3 PUFA. Secondly, we examined the association between genetic variants in the 5-LOX pathway and MI, a major inflammatory pathway, involved in the metabolism of AA and EPA. Selected single nucleotide polymorphisms (SNP) and haplotypes were explored. Lastly, we explored the diet-gene interaction between substrates for the 5-LOX pathway (EPA, AA) and a tandem repeat polymorphism in the 5-LOX pathway in relation to the risk of incident MI.

CHAPTER 2. AIMS AND HYPOTHESES

Study I

Aim: To examine the association of dietary fish consumption with incident MI in a cohort study using a semi quantitative food frequency questionnaire.

Hypothesis: Consumption of fish, in particular intake of fatty fish, is negatively associated with MI. Further, the intake of marine n-3 PUFA is also negatively associated with MI.

Study II

Aim: To investigate the association between adipose tissue content of marine n-3 PUFA and incident MI.

Hypothesis: The content of total and individual marine n-3 PUFA (EPA, docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA)) is negatively associated with MI.

Study III

Aim: To explore associations between 22 SNPs and one tandem repeat polymorphism, from four candidate genes, encoding key enzymes in the 5-LOX pathway and incident MI. Individual SNPs and haplotypes will be assessed.

Hypothesis: Variant genotypes are associated with MI compared with carriers of the wildtype.

Study IV

Aim: To explore diet-gene interaction between a tandem repeat polymorphism in the *ALOX-5* gene (rs59439148) and adipose tissue content of EPA or AA with incident MI.

Hypothesis: Homozygous carriers of the variant alleles will have a higher risk of MI compared with carriers of wildtype alleles, and EPA will attenuate the associations, while AA will exacerbate the associations.

CHAPTER 3. BACKGROUND

3.1. ATHEROSCLEROSIS AND CHD

CHD refers to the diseases of the coronary arteries that supply oxygen-rich blood to the myocardium (1). CHD is almost exclusively caused by the development of atherosclerotic plaques in the coronary arteries that, over time, can cause a narrowing of the arterial lumen and thereby a limitation of the blood flow to the myocardium. CHD may also present clinically as an acute event, caused by the sudden rupture of a plaque, resulting in the abrupt limitation of the blood flow by an intraluminal thrombus and subsequently, myocardial ischemia and possibly myocardial infarction (MI) (2,3). For a comprehensive definition of MI, see (4).

Atherogenesis and the formation of atherosclerotic plaques constitute a complex process situated in the arterial wall, as reviewed by several authors (2,3,5–9). The initial steps of atherogenesis involve irritative stimuli (e.g. dyslipidemia, hypertension, diabetes mellitus, tobacco smoking etc.) leading to the activation of vascular endothelium cells, facilitating the migration of leucocytes and LDL particles into the arterial wall (7). This cell migration and accumulation of cholesterol leads to the initiation of a local inflammatory response that in turn further activates endothelial cells and smooth muscle cells and attracts more inflammatory cells. This results in a vicious circle with persisting inflammation (9). As the atherosclerotic plaque develops, the accumulation of foam cells forms a lipid-rich core consisting of lipids, inflammatory cells and cellular debris from the resulting cellular apoptosis. The activation and proliferation of smooth muscle cells in the intima of the arterial wall, on the other hand, form a fibrous cap that seals the lipid-rich core from the vascular lumen (2).

In advanced atherosclerotic plaques, cell proliferation and accumulation of lipids may result in clinical manifestations of the growing plaque. Usually, these manifestations are caused by a gradual narrowing of the vascular lumen, resulting in limited blood flow, and ischemia. Clinical manifestations may also involve plaque rupture and thrombus formation, but interestingly, it is not necessarily the flow-limiting or largest plaques that are associated with rupture and thrombus formation (2). Naturally, a lot of attention has been given to identify vulnerable plaques that seem to be characterised by a thin fibrous cap with little collagen tissue and few smooth muscle cells in the cap but, in contrast, a high number of macrophages and other inflammatory cells. It has been suggested by several authors that inflammation might be important to the stability of the fibrous cap and hence the risk of plaque rupture and acute events (8,9).

3.2. MARINE N-3 PUFA AND CHD

Classification of marine n-3 PUFA and n-6 PUFA

Fatty acids can be classified as saturated, monounsaturated or polyunsaturated according to the number of double bonds on the carbon chain (Figure 1). Furthermore, the configuration around the double bonds can be either cis or trans configuration, and while most naturally occurring fatty acids are in the cis configuration, trans fatty acids are produced from industrialised processing (apart from ruminant trans fatty acids).

N-3 PUFA, also known as omega-3 PUFA, belong to a group of essential fatty acids together with n-6 PUFA which implies that these fatty acids are necessary to maintain normal body functions but cannot be adequately synthesised endogenously (10,11). They are characterised as n-3 or n-6 PUFA by the position of the first double bond from the methyl end at carbon atom number 3 or 6, respectively.

N-3 PUFA can be divided into two groups of different biological origin: plants and seafood. Thus, alpha linolenic acid (ALA) with 18 carbons and 3 double bonds (C18:3n-3) is mainly derived from plants, while the major marine n-3 PUFA: EPA (C20:5n-3), DPA (C22:5n-3) and DHA (C22:6n-3) (Figure 1) are mainly derived from seafood (10,11). The marine derived n-3 PUFA are sometimes termed long chain n-3 PUFA to differentiate them from ALA. ALA can, to a limited extend, be converted to EPA (12), and may have beneficial effects in relation to CHD by itself (13,14). In humans, EPA and DHA can be interconverted via DPA through elongation and desaturation or vice versa. While EPA and DHA are considered the biologically most important with regard to cardioprotective effects, little is known about the biological effects of DPA. In this report, n-3 PUFA are used as an abbreviation for marine n-3 PUFA unless otherwise specified.

N-6 PUFA consist mainly of linoleic acid (LA, C18:2n-6) that can be converted to AA (20:4n-6). The main dietary sources of LA include most vegetable oils, eggs and, for AA, meat and dairy products (10,15).

The role of n-6 PUFA in CHD has been debated. Most observational studies and dietary intervention trials have fund a neutral or slightly beneficial effect of n-6 PUFA intake (16,17). However, some biomarker studies on adipose tissue content of AA, evaluating the endogenous exposure to AA specifically, have found a positive association with MI (18,19), while others did not support these findings (20,21). This has raised concerns about n-6 PUFA being harmful to cardiac health by potential pro-inflammatory and pro-thrombotic effects, possibly mediated via AA and the production of eicosanoids (22–24).

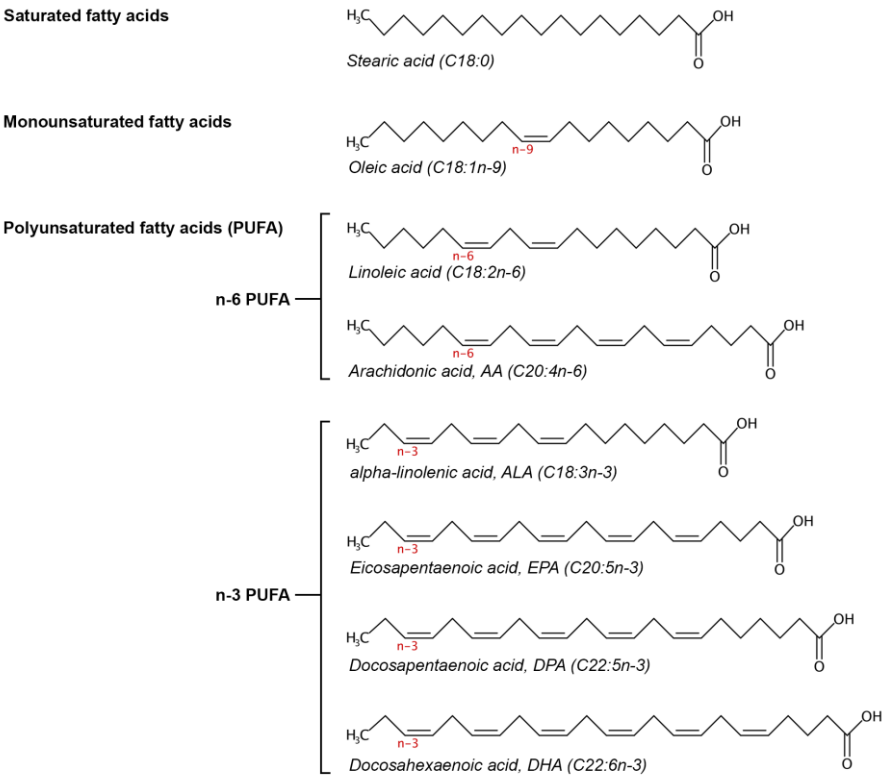

Figure 1. Fatty acids, classification according to the number of double bonds. Examples of saturated, monounsaturated and polyunsaturated fatty acids. ALA is derived from specific plant oils, while EPA and DHA are mostly obtained from fish and seafood.

Marine n-3 PUFA in CHD

More than 40 years ago, doctors from Aalborg Hospital, H.O. Bang and J. Dyerberg, suggested that seafood, in particularly EPA, might reduce the risk of atherothrombosis (25). This was based on studies in Greenland Eskimos, showing a low prevalence of MI in this population along with an anti-atherogenic lipid profile and markedly reduced platelet reactivity compared to controls living in Denmark (25–27). Bang and Dyerberg attributed their findings to the diet of the Eskimos based on seafood, in particular seal and whale, with an extremely high content of n-3 PUFA (25,28).

This led researchers worldwide to explore the hypothesis of a beneficial effect of n-3 PUFA on CVD, and in particular CHD. In general, early studies supported the hypothesis (11,29–31) and overall, most epidemiological studies have suggested an inverse association between fish consumption and the risk of CHD (29–38), although not all studies have reported this (39,40). The evidence seems to be stronger towards

an inverse association of fish consumption with fatal CHD and sudden cardiac death, than non-fatal coronary events. A large meta-analysis by He et al. from 2004 concluded that fish consumption was inversely associated with the incidence of fatal CHD in a dose-dependent manner (41).

Epidemiological studies have been followed by a number of clinical trials with n-3 PUFA supplements as secondary prevention of CHD. In 1989, the Diet And Reinfarction Trial (DART) was published (42) where 2,033 patients with MI received different dietary advice, including fish consumption twice a week. After a 2-year period, the group receiving advice of increased fish intake had a 29% reduction in overall mortality compared to controls. Later the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico (GISSI) Prevenzione trial was undertaken, including 11,324 post MI patients (43). A daily supplement with 1 g n-3 PUFA (given as one fish oil capsule containing 0.85 g of EPA+DHA) reduced total mortality, CHD mortality and sudden cardiac death (43,44). Based on these and other trials, several scientific communities now recommend that patients with CHD should eat at least 1 g of n-3 PUFA daily to reduce further cardiovascular events (45,46). However, recent intervention trials have been less convincing towards a positive effect on CHD prevention, and no significant association was demonstrated between intake of n-3 PUFA supplements and CHD (47–50). Recently, two meta-analyses was published, reporting a more modest effect of n-3 PUFA supplements and fish consumption than earlier trials have suggested (51,52). Additionally, a meta-analysis on cohort studies using biomarkers of n-3 PUFA, reported modest effects for EPA, DPA and DHA with relative risks around 0.90 for fatal CHD (53).

A number of different mechanisms have been proposed to explain the cardioprotective effects of n-3 PUFA. These include effects on the vessel wall, haemostasis, plasma lipids and the immune system (Table 1). Some of these mechanisms may be complementary, and the biology of n-3 PUFA may be complex and almost certainly mediated through a combination of mechanisms (11,14). Trials have reported that n-3 PUFA supplements decrease plasma triglycerides in a dose-dependent manner (54,55) and might reduce the fraction of atherogenic small dense LDL particles (56). Indeed, a dose-dependent reduction on triglycerides is perhaps the best documented effect of marine n-3 PUFA, and in light of the current focus on remnant lipoproteins in atherogenesis, this might be of importance. Studies also suggest an antithrombotic effect of n-3 PUFA supplements, by reducing platelet reactivity and switching the production of thromboxanes and prostaglandins towards a more anti-aggregatory profile (11,57–59). It has also been suggested that n-3 PUFA prevent arrhythmias (44,60) and increase heart rate variability (61), which might be an important mechanism in explaining an effect of n-3 PUFA on sudden cardiac death. Finally, it has been demonstrated that n-3 PUFA are incorporated into atherosclerotic plaques, and a study by Thies et al. showed that a supplement of 1.4 g/day n-3 PUFA, prior to carotid endarterectomy, resulted in thickening of the fibrous cap and less macrophage infiltration in carotid plaques (62) suggesting increased plaque stability.

Atherosclerosis has indeed been recognised as a multifactorial disease involving inflammatory processes (5,6), and thus the immunomodulatory properties of n-3 PUFA might be important in this respect.

Table 1. Proposed mechanisms for the cardioprotective effects of n-3 PUFA.

- Anti-atherosclerotic
- Anti-inflammatory
- Anti-aggregatory
- Lowering of platelet reactivity
- Lowering of triglycerides
- Anti-arrhythmic
- Lowering of blood pressure

Despite a fair amount of clinical trial evidence together with animal and in vitro experiments, the role of n-3 PUFA in the prevention of CHD is still debated. Notably, important discrepancies exist between secondary prevention trials and epidemiological studies from a primary prevention point of view and furthermore there is a gap between the findings from early trials compared with more recent trials. For reviews on n-3 PUFA and its role in CVD see (11,14,52,63–68).

3.3. THE 5-LIPOXYGENASE PATHWAY AND CHD

Biosynthesis of leukotrienes

Leukotrienes are known as highly pro-inflammatory lipid mediators and belong to a group of biochemically active substances called eicosanoids, also including thromboxanes, prostaglandins and lipoxins, among others. As the name implies, the leukotrienes are mainly produced by leukocytes, in particular monocytes, granulocytes, mast cells and dendritic cells (69,70). When leukocytes are activated, AA is liberated from cell membrane phospholipids by phospholipase A_2. Subsequently, leukotrienes are produced via LOX enzymes where 5-LOX, one of six different LOX pathways found in humans, has been associated with inflammatory disease and CHD (69). The 5-LOX pathway consists of four key enzymes, where arachidonate 5-lipoxygenase (ALOX-5) and the 5-lipoxygenase activating protein (FLAP) constitute the first enzymatic step that metabolise AA and EPA. ALOX-5 constitutes the enzymatic activity but is dependent on activation by FLAP. This first enzymatic step is also the rate limiting step in the pathway. By a two-step process AA is oxidised by the enzymatic complex into an intermediary product, leukotriene A_4 (LTA_4). LTA_4 is highly unstable and therefore rapidly converted by either leukotriene C_4 synthase (LTC_4-S) or leukotriene B_4 hydrolase (LTB_4-H) resulting in the formation

of either cysteinyl leukotrienes (CysLT) or leukotriene B_4 (LTB_4), respectively (Figure 2) (69). While AA is the most abundant in membrane phospholipids, EPA serves as an alternative substrate for LOX enzymes, and because of this biochemical resemblance with AA, EPA functions as a competitive inhibitor of AA metabolism (71). Interestingly, leukotrienes derived from EPA are substantially less potent, and thus leukotrienes derived from EPA are generally far less pro-inflammatory compared to corresponding leukotrienes from AA (72,73). E.g. leukotriene B_5 (LTB_5) derived from EPA was shown to be more than 30 times less active as chemoattractant compared to LTB_4 derived from AA (73).

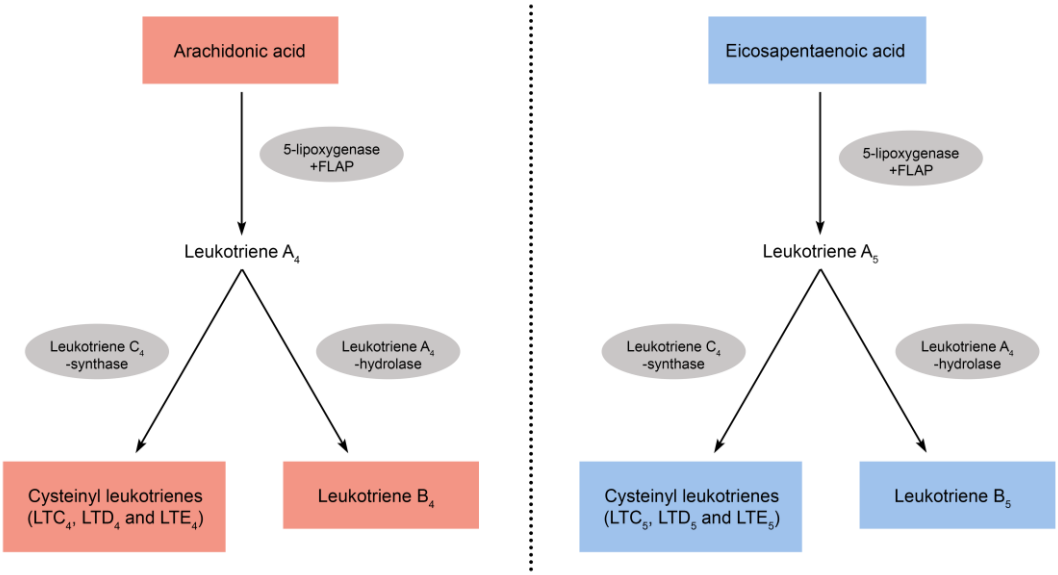

Figure 2. Schematic outline of the 5-lipoxygenase pathway. Arachidonic acid (AA), or alternatively eicosapentaenoic acid (EPA), is metabolised by 5-lipoxygenase (5-LOX) enzymes forming different leukotrienes. In the first enzymatic step, AA or EPA is oxidised by an enzymatic complex consisting of arachidonate 5-lipoxygenase and an activating protein (FLAP), forming an unstable intermediate (leukotriene A). Next, leukotriene A is rapidly converted to either leukotriene B or the cysteinyl leukotrienes by either leukotiene A_4-hydrolase or leukotriene C_4-synthase, respectively. Importantly, the 5-series leukotrienes derived from EPA are far less pro-inflammatory compared to the 4-series leukotrienes derived from AA.

Linking the 5-LOX pathway and atherosclerosis

Evidence suggesting a link between the 5-LOX pathway along with the bioactive leukotrienes and the development of atherosclerosis and atherothrombotic disease has been presented at multiple levels. For recent reviews see (69,74–77). Evidence includes animal, in-vitro and human studies. Thus, in a mouse model prone to atherosclerosis, it was demonstrated that the knock-out of the *ALOX-5* gene led to a high resistance against development of atherosclerosis (78). Furthermore, in a similar mouse model, another study reported a decrease in atherosclerosis from inhibition of

FLAP (79). Other aspects of the 5-LOX pathway have been implicated in atherosclerosis traits in animal studies, including the leukotriene B4 receptor (80,81).

Human studies have confirmed the presence of 5-LOX enzymes in atherosclerotic plaques, and interestingly, Spanbroek et al. showed that levels of ALOX-5 were higher in more advanced plaques (82). Furthermore, high levels of ALOX-5 and LTA_4-H in human plaques have been associated with symptoms of plaque instability (83,84), suggesting a key role of the 5-LOX pathway in late stages of atherosclerosis and acute atherothrombotic events.

Leukotrienes produced from the 5-LOX pathway promotes inflammation which may be important in atherothrombotic disease as it may enhance plaque progression and affect the stability of plaques. LTB_4 is among the strongest known chemotactic agents, and as such, it may mediate monocyte infiltration into the atherosclerotic plaque (80,85). Actions of LTB_4 are mediated through the BLT1 and BLT2 receptors that have been found on most types of leukocytes found in atherosclerotic lesions (69,85,86). BLT receptors are normally present at low levels in the resting endothelial cell, but activated vascular endothelium has been reported to upregulate BLT-receptor expression, and LTB_4 itself has been shown to induce this upregulation which may suggest an involvement of LTB_4 in early stages of atherogenesis (69,86). Additionally, smooth muscle cells and endothelial cells in atherosclerotic plaques also express BLT receptors. Thus, it is plausible that LTB may exert their biological effects via the BLT receptors in the setting of atherosclerosis. CysLT elicit their biological actions via two CysLT receptors (CysLT-1 and -2) which are present on most leukocytes, in particular mast cells, but are also found on smooth muscle cells (85,86). CysLTs are well known mediators of inflammation in asthma, however, CysLT has also been implicated in atherosclerosis where they have been suggested to increase vascular permeability and enhance inflammation (70,85). Furthermore, CysLT have been associated with vasoconstriction in atherosclerotic coronary arteries but not in normal coronary arteries without atherosclerosis (87).

In summary, the 5-LOX pathway is present in vascular lesions and probably upregulated concurrently with plaque advancement. Leukotrienes elicit a number of pro-inflammatory effects, and evidence suggests that leukotrienes are involved in leukocyte migration, activation and proliferation in atherogenesis. Inflammatory stimuli in general have been suggested to play an important role in plaque development and in destabilisation of plaques. Figure 3 summarises the suggested involvement of the 5-LOX pathway and leukotrienes in atherogenesis.

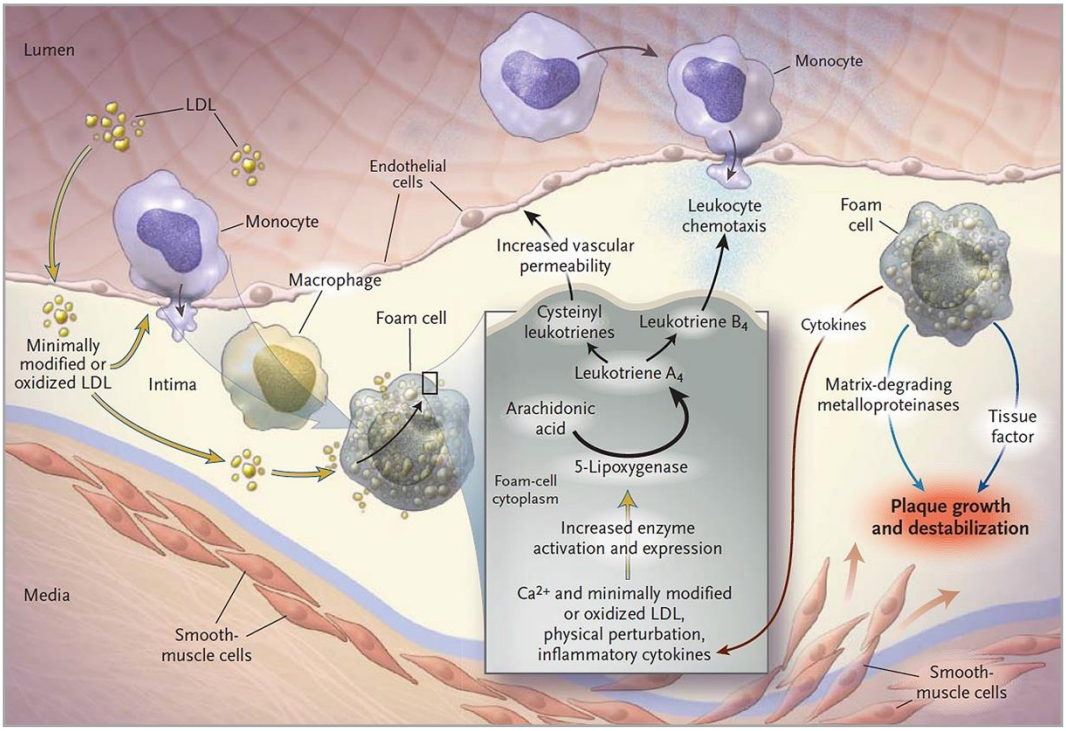

Figure 3. Suggested roles of leukotrienes in the development of atherosclerosis. Monocytes enter the vascular endothelium, and a cascade of inflammatory stimuli activates monocytes that develop into foam cells. The 5-LOX pathway in activated leukocytes produces leukotrienes among other pro-inflammatory substances that promote inflammation and further enhance leukotriene production, creating a vicious circle. *Reproduced with permission from (De Caterina et al. N Engl J Med 2004; 350:4-7), Copyright Massachusetts Medical Society.*

3.4. GENETIC POLYMORPHISMS IN THE 5-LOX PATHWAY

Initial animal and human biochemical studies fuelled the search for genetic associations between 5-LOX polymorphisms and CVD endpoints. A number of epidemiological studies have been undertaken examining the four candidate genes, encoding the key enzymes directly involved in the 5-LOX pathway.

ALOX-5. Most attention has been focused on *ALOX-5* and *FLAP*, the rate-limiting step in the pathway. Thus, Dwyer et al. (88) examined a tandem repeat polymorphism in the promoter region of *ALOX-5*, containing a varying number of SP1 transcription factor binding motifs (5'-GGGCGG-3') and found variant alleles to be associated with higher intima-media thickness of the carotid arteries, a marker of atherosclerosis, compared to carriers of two wild type alleles. This polymorphism has been

investigated in a number of other studies with different endpoints, including ischemic stroke and MI (89–93). Results have been inconsistent, but interestingly, significant diet-gene interaction between 5-LOX substrates and the tandem repeat has been reported by two studies (88,89). Other polymorphisms in ALOX-5 have also been examined. Thus, two studies examined a number of SNPs designed to cover the genetic variation across *ALOX-5* (92,94), but no consistent associations with CVD endpoints, except for SNPs closely linked to the tandem repeat, were reported.

FLAP. The deCODE investigators were the first to highlight 5-LOX genes in a genome-wide association study (95–97). Thus, *FLAP* was identified as an important gene involved in atherothrombotic disease. The group reported two haplotypes (Hap-A and Hap-B) that were associated with higher risk of MI and stroke among carriers in two independent cohorts (Icelandic (Hap-A) and British (Hap-B)) (95). Later, these results were replicated in a Scottish population with support for Hap-A but not for Hap-B (96). Following the first studies by the deCODE investigators, other studies have investigated *FLAP*, genotyping the SNPs selected by the deCODE investigators. Some studies supported the associations (98–102), while others did not find these haplotypes to be associated with the risk of MI or stroke (92,94,103,104). Most studies found associations for haplotypes rather than individual SNPs, and though few studies also reported associations for SNPs, no consensus has been obtained pointing towards a functional polymorphism.

LTA4-H. The deCODE investigators also investigated the LTA4-H gene and identified a risk haplotype (Hap-K) for MI (97). This haplotype was tested by other investigators (92,104) supporting the findings from the Islandic cohort. In a study by Zhao et al. (105), the same SNPs as selected by the deCODE group were studied, but no association between carrier status of Hap-K and carotid intima-media thickness was found. However, the group defined a new haplotype (Hap-E) that was associated with a lower risk of atherosclerosis among carriers compared with non-carriers. The same group tested interaction between intake of n-3 and n-6 PUFA with Hap-E in a population of American Indians, and reported a significant diet-gene interaction for this haplotype (106).

LTC4-S. In *LTC4-S,* two specific SNPs (rs730012 and rs3776944), located close to the promotor region of the gene, have been investigated. A Danish cohort study by Freiberg et al. found rs730012 variants negatively associated with ischemic stroke, while rs3776944 was positively associated with the risk of stroke (107). However, this could not be confirmed in two other studies where rs730012 was positively associated with markers of atherosclerosis and ischemic stroke (108,109).

CHAPTER 4. METHODS

4.1. STUDY POPULATION

The Diet, Cancer and Health study

The Diet, Cancer and Health study is a prospective cohort study with the primary objective of analysing the etiological role of diet in the development of cancer (110). Eligible participants were born in Denmark, living in the urban areas of Copenhagen and Aarhus and not registered with a cancer diagnosis in the Danish Cancer Registry at the time of invitation. In total, 160,725 persons aged 50-64 years were invited between December 1993 and May 1997. A letter of invitation describing the study was sent to each potential participant, and if invitees did not respond after three weeks, one reminder was sent after which no further attempt was made to recruit the subject. A unique ten digit personal registration number for each subject was retrieved from the Civil Registration System to establish future linkage between eligible subjects and National Registries. A total of 57,053 (35%) accepted the invitation and were enrolled into the study. Participants were followed until the end of July 2013.

For the studies included in this thesis, we excluded participants if a cancer diagnosis was reported, that was not already recorded in the cancer registry at the time of invitation, in line with the intention-to-include criteria. We also excluded participants registered with a predefined endpoint (previous MI or cardiac arrest).

From the cohort, a randomly selected sample of 3,500 participants was drawn. This made up the "sub-cohort" that was used to represent the cohort in nested case-cohort designs. This strategy was used when including adipose tissue biopsies and information on genotype in the analyses (study II-IV). In study I analyses were based on the entire cohort. Figure 3 illustrates the selection process for all four studies included in this thesis.

The study was conducted in accordance with the Helsinki Declaration and approved by the regional ethics committees.

Baseline questionnaires

At baseline, each participant filled in two detailed questionnaires, a background and a dietary questionnaire. To ensure that unclear or missing responses were minimised, both questionnaires were checked with an interviewer.

The background questionnaire included detailed information on socio-economic status, e.g. type and length of education, line of occupation. A variety of lifestyle factors were recorded, such as smoking status / tobacco use, alcohol consumption, physical activity, educational level and occupation. Furthermore, health information

was collected including family history of CVD, disease diagnosed by a physician and use of medication with focus on lipid lowering, anti-hypertensive and anti-diabetic drugs.

The dietary questionnaire comprised of a detailed 192-point semi-quantitative food frequency questionnaire, including 26 specific questions regarding intake of fish and food products containing fish, at baseline. The questionnaire has been validated and described in detail elsewhere (111). Participants were asked to estimate their daily intake of foods in natural units such as pieces of fruit, slices of bread and glasses of different drinks. For mixed dishes and meals, sex-specific portion sizes were calculated using data from a calibration study. By multiplying the frequencies of intake by the portion size, an individual average intake in g/day of all foods and nutrients was calculated. Different species of fish were categorised as either lean or fatty according to their content of marine n-3 PUFA, below or above 1 g/100 g. In the Diet, Cancer and Health study cohort, fatty fish intake was mainly comprised of herring, salmon/trout and mackerel, while lean fish was comprised mainly of plaice/flounder and cod (36). The intake of herring in the Danish population is particularly high compared to most other countries, whereas the intake of other species, e.g. sardines, were relatively low. The dietary intake of specific nutrients, including total and individual marine n-3 PUFA (EPA, DPA and DHA) was calculated using the software FoodCalc (112) based on Danish food composition tables.

Outcome assessment

We identified all participants in the cohort who were registered with an incident diagnosis of MI in the Danish National Patient Registry (NPR) and/or the Danish Causes of Death Registry (CDR), according to the International Classification of Disease (ICD) 8 (410.00-410.99) or ICD-10 (I21.0-I21.9) coding, during the study period (Figure 4). Furthermore, all cases of cardiac arrest (ICD-8: 427.27 or ICD-10: I46.0-I46.9) were included if the arrest was considered to be of cardiac origin after validation. The NPR and the CDR hold information on diagnoses and procedures, and causes of death, respectively. Both registries classify diseases according to the ICD. The ICD 8th edition was used until January 1995, when the ICD 10th edition was implemented. In the NPR, discharge diagnoses from in-hospital patients have been registered since 1977, and since 1995 diagnoses from emergency rooms and out-patient visits have been recorded as well. At present, the NPR has been updated until the 31st July 2013, and the CDR has been updated until the 31st of December 2011. A previous study validated the diagnoses of first-time MI until 31st of December 2003 (113). From this study, an algorithm was established to evaluate new cases from January 2004 to July 2013.

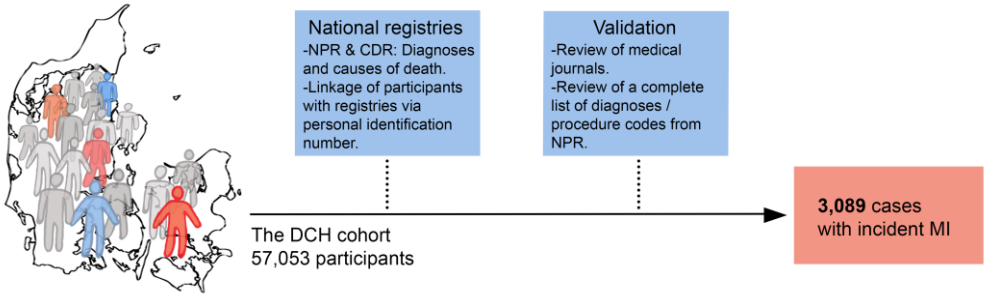

Figure 4. Case identification. Flow chart illustrating the identification and validation of cases. By personal identification numbers we identified all participants with a diagnosis of incident myocardial infarction (MI) or cardiac arrest from two national registries (NPR & CDR), and secondly, all cases were validated by review of medical journals or by an algorithm.

The algorithm takes advantage of the previously mentioned validation study that found a high positive predictive value for the diagnosis of MI when recorded as in-hospital cases (*PPV*=92,4%), and therefore all new cases with the diagnoses of I21.x (MI), given from a ward, registered in the NPR were readily accepted as new cases of MI. Potential cases with an I21.x diagnosis from out-patient clinics and emergency rooms or the CDR and the I46.x (cardiac arrest) which potentially could represent a fatal MI were validated. The validation was performed using an Excel spreadsheet containing all diagnoses and interventional procedures recorded in the NPR for each potential case. Each subject was evaluated individually, and it was decided whether the subject could be considered a potential case or not. Our point of view was that every subject with an I21 or I46 diagnosis was a potential case of MI unless data suggested otherwise. For non-fatal events, the registered events/procedures could, in most cases, clarify the diagnosis, but for fatal events we could only rely on information prior to the event. For each potential case it was decided whether the diagnosis was highly likely or "just" possible. Possible cases included potential cases that could not be ruled out as cases but neither could be regarded as a highly likely case. Thus, some misclassification was inevitable, but most likely, misclassification of non-cases as possible cases would tend to blunt the association between the intake/adipose tissue content of n-3 PUFA and incident MI. Both probable and possible cases were included as cases (n=332). In summary, 1,670 subjects were accepted as cases without validation because the diagnoses of MI were given by a ward, while 636 potential cases were validated, of which 332 were classified as cases whereas 304 were classified as non-cases (Table 2).

Table 2. Case validation after December 2003 based on the validation study by Joensen et al. (113)**.**

	Validation	No validation	Total
Non-case	304	-	304
Case	332	1,670	2,002

4.2. ADIPOSE TISSUE ANALYSES (STUDY II-IV)

An adipose tissue biopsy was taken from the buttocks of all participants using a luer lock system (Terumo, Terumo Corp, Tokyo, JP) consisting of a needle, a venoject multisample luer adaptor, and an evacuated blood tube, according to the method of Beynen and Katan (114). Samples were flushed with nitrogen and stored at -150°C until analysis. When analysed, biopsies were thawed and preheated at 50°C for 10 min. Subsequently, the fat was dissolved in heptane at 50°C, and fatty acids were transesterified by 2 mol/L KOH (potassium hydroxide) in methanol at 50°C for 2 min, according to IUPAC standard methods for analysis of oils, fats, and derivatives. Fatty acid composition was determined by gas chromatography using a Varian 3900 GC with a CP-8400 autosampler (Varian, Middleburg, NL) equipped with a flame ionization detector. Split injection mode, a CP-sil 88, 50 m x 0.25 mm ID capillary column, temperature programming from 90°C to 210°C, and constant flow were used. Helium was used as carrier gas. Commercially available standards (Nu-chek-Prep, Inc., Minnesota, US) were used to identify the individual fatty acids. The method has been described in detail previously (35).

The content of fatty acids were expressed as weight percent of total fatty acids, and the inter assay coefficients of variation were 6.4%, 3.5%, 4.1% and 3.2% for EPA, DPA, DHA and AA, respectively. Analyses of adipose tissue biopsies were performed for all cases of MI and a randomly selected sub-cohort (n=3,500).

4.3. GENOTYPING (STUDY III-IV)

Selection of SNPs and DNA extraction

From a review of the current literature, we selected four candidate genes to examine the 5-LOX pathway (*ALOX-5, FLAP, LTA4-H* and *LTC4-S*). Next, 22 SNP markers were selected based on previously reported associations with CVD, with preference for CHD, and a confirmed minor allele frequency (MAF) > 0.05 in Caucasians.

From whole blood, DNA was extracted using Kleargene^{TM} XL DNA extraction kit (LGC Genomics, Queens Road, Teddington, Middlesex, UK). The Kleargene method is based on detergent-driven cell lysis, followed by guanidinium isothiocyanate-mediated DNA binding to silica. Next, contaminants were removed by washing and DNA was subsequently eluted into a low salt buffer. Extracted DNA was stored at -20°C. DNA extraction was performed for all cases of MI and a randomly selected sub-cohort (n=3,000).

Sequencing

The tandem repeat polymorphism was analysed by microtitre plate (MTP)-sequencing technique, using standard 96-well plates. PCR-products were prepared from genomic DNA, using MyTaq™ DNA polymerase (Bioline US Inc.) along with the following primers:

5'-TCAGGAGAGAACGAGTGAAC-3' (forward)
5'-GTCCAGGTGTCCGCATC-3' (reverse).

Forty reaction cycles were performed at 55°C. From the PCR-products, sequencing was done using an ABI 3730XL DNA analyser (Thermo Fischer Scientific Inc.), and Chromatograms were interpreted by a trained laboratory technician, identifying the number of tandem-repeats for each allele. In case of uncertainty, the chromatograms were rechecked by another technician, and results were discussed and agreed upon.

KASP genotyping analyses

SNP genotyping was performed by LGC Genomics using the commercially available KASP™ genotyping assay. KASP is based on a competitive, allele specific PCR genotyping technique with a homogenous fluorescent-based reporting system (115). The KASP Primer mix, containing the allele specific forward primers and a universal reverse primer, was mixed with the KASP Master mix, containing the taq polymerase enzyme and the passive reference dyes, FAM™ and HEX™. The reaction mix was aliquoted to standard 96-well plates containing DNA samples from the study cohort, and at least one "no template control" per plate. PCR was performed, and the fluorescent signal was analysed using a BMG PHERAstar plate reader (BMG Labtech Ltd., Aylesbury, UK). The analysis was performed according to the protocol provided by LGC Genomics (116). SNP alleles correspond to the positive/forward DNA strand according to dbSNP, human assembly GRCh38.p2 (117).

4.4. STATISTICS

The endpoint in all the studies was time to incident (first-time) MI. Time-to-event data were analysed in a cohort design (Study I) using Cox proportional hazards multivariate regression models with age as the time axis and delayed entry. Age was regarded as the most important time related risk factor for MI and therefore used as time axis to properly adjust for this co-variate in the models. Participants were treated as "at risk" from baseline until endpoint, death, emigration or end of follow-up occurred.

For Study II-IV we used a nested case-cohort design. To account for the smaller subsample of the cohort, we used a weighting scheme, assigning weights to each non-case member of the sub-cohort, and robust variance estimates were calculated as described by Kalbfleisch and Lawless (118).

Analyses were conducted for the entire cohort and stratified by sex whenever considered methodologically appropriate. The proportional hazards assumption was checked by visual inspection of log-log plots and by graphical evaluation of scaled

Schoenfeld residuals, with no significant violations. P-values (two-tailed) <0.05 were considered statistically significant. The latest version of STATA (StataCorp, College Station, TX, US) was used as statistical software.

Model selection

A large number of risk factors have been documented and proposed for MI. A priori, we selected co-variates to include in the adjusted models based on the current evidence of association with MI and/or the exposure(s). Adjustment for potential confounding was applied in different steps allowing for clear interpretations of the results. We used the same strategy to control confounding for all studies, as outlined below. However, for study III with genotype as the primary exposure, we considered the more basic models as the most appropriate (model A1 and A2). All continuous variables were included in the models as restricted cubic splines with five knots.

Model A1. Crude models were presented without adjustment for confounders, except for the pooled analyses including both women and men where we adjusted for sex. Large variations between the crude and adjusted models could indicate the importance of confounding.

Model A2. In model A2, we adjusted for traditional epidemiological risk factors for MI, included the following variables:

- Smoking habits, categorised as never, former or current (<15 g/day, 15-25 g/day, >25 g/day) smokers
- Physical activity (hours/week) of moderate to high intensity
- Educational level, categorised as basic school (<7 years of education), higher education (8-10 years, >10 years)
- Alcohol intake (g/day)
- Body mass index (kg/m^2)
- Waist circumference (cm)
- Menopausal status (pre- or post-menopausal) for women.

In general, this model has a clear interpretation and reduces potential confounding by adjusting for important lifestyle and demographic determinants.

Model B. In addition to the variables included in model A2, we applied a second layer of variables adjusting for medical history. Variables were self-reported history of physician-diagnosed disease and use of specific medications related to the diseases.

- Hypertension and/or use of anti-hypertensive medications
- Hypercholesterolaemia and/or use of lipid-lowering drugs
- Diabetes mellitus (all types).

These variables are normally considered important risk factors for MI and potential confounders. However, considering the broad range of mechanisms proposed for n-3 PUFA, including effects on blood pressure, plasma lipids and inflammation, these co-

variates may represent intermediate steps in causal pathways by which n-3 PUFA may affect the risk of MI. Thus, including these additional variables may introduce bias by eliminating the causal pathway through the mediators. However, the potential mediators are probably only affected by the exposure to a limited extent and residual confounding may be the implication if not including these variables, particularly since they represent some of the strongest risk factors for MI.

Model C. Here, we included dietary variables in addition to the traditional risk factors from model A2. This implies adjustment for potential confounding from other aspects of the diet, but it may also introduce dietary patterns which are less relevant compared with an ordinary diet. For this reason, we limited the number of dietary co-variates to the most relevant ones based on known association with MI. Furthermore, variables were selected based on the nature of the exposure. E.g. when using specific fatty acids, we included co-variates at the level of specific nutrients, while complex whole food elements were used when evaluating fish intake. Thus, dietary co-variates reflected the nutritional level of the exposure.

CHAPTER 5. STUDIES

This thesis is based on four studies that are included as published papers or manuscripts in the appendix. This section is meant to provide a short overview of the studies and present additional figures/tables which were not included in the final version of the manuscripts. For a more detailed description of the applied methods, please refer to "Chapter 4. Methods" or the full version of the manuscripts.

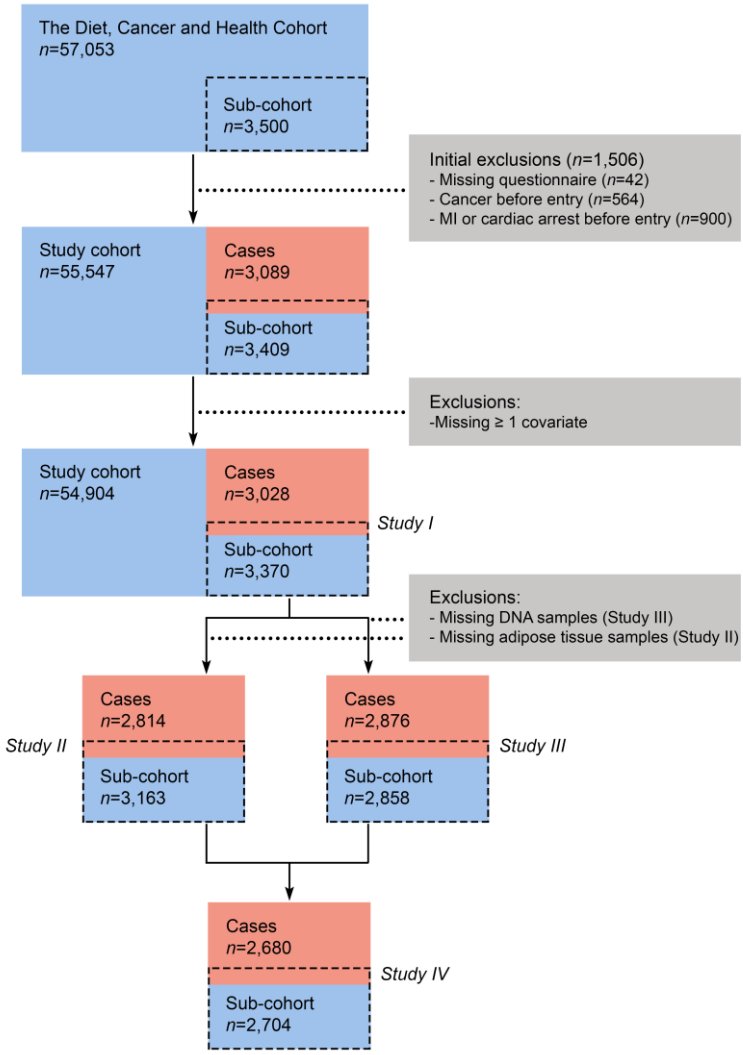

Figure 5. Cohort selection process - overview of the four studies.

Table 3. Key baseline characteristics for the cohort, sub-cohort and cases

Variable	Men						Women					
	Cohort		*Sub-cohort*		*Cases*		*Cohort*		*Sub-cohort*		*Cases*	
Sex (%)	47.2	(25,913)	53.4	(1,800)	70.5	(2,136)	52.8	(28,991)	46.6	(1,570)	29.5	(892)
Age (years)	55.9	(51.2;63.3)	56.3	(51.2;63.3)	57.7	(51.7;63.9)	56.2	(51.2;63.2)	56.3	(51.0;62.9)	59.3	(52.4;64.2)
Physical activity (h/week)	2.0	(0.0;8.0)	2.5	(0.0;8.0)	2.0	(0.0;8.0)	2.5	(0.0;8.0)	2.5	(0.0;8.0)	2.0	(0.0;7.0)
BMI (kg/m2)	26.1	(22.5;31.1)	26.3	(22.6;31.1)	26.9	(23.2;32.2)	24.8	(20.8;31.2)	24.8	(21.0;31.4)	25.9	(20.9;33.3)
Waist circumference (cm)	95.0	(84.0;108.5)	95.0	(85.0;108.0)	97.0	(86.0;112.0)	80.0	(69.0;97.0)	80.0	(69.0;98.0)	84.0	(70.0;102.0)
Smoking (% (n))												
- Never smoker	26.1	(6,772)	26.7	(480)	18.2	(389)	43.9	(12,719)	43.5	(683)	27.1	(242)
- Former smoker	34.3	(8,892)	34.9	(628)	29.5	(631)	23.5	(6,797)	22.0	(346)	19.4	(173)
- <15 g/day	10.6	(2,744)	10.8	(195)	12.4	(265)	15.3	(4,421)	16.1	(252)	22.2	(198)
- 15-25 g/day	17.5	(4,522)	16.8	(302)	23.8	(509)	14.9	(4,305)	15.8	(248)	26.1	(233)
- >25 g/day	11.5	(2,983)	10.8	(195)	16.0	(342)	2.6	(748)	2.6	(41)	5.2	(46)
Educational level (% (n))												
- Basic school	34.2	(8,867)	34.1	(614)	43.2	(923)	31.2	(9,043)	31.2	(490)	44.0	(392)
- Higher education. 1-3 years	41.7	(10,814)	41.0	(738)	37.3	(796)	50.2	(14,556)	49.8	(782)	46.3	(413)
- Higher education. >3 years	24.1	(6,232)	24.9	(448)	19.5	(417)	18.6	(5,392)	19.0	(298)	9.8	(87)
Menopausal status (%)												
- Post-menopausal	-	-	-	-	-	-	58.6	(16,975)	58.4	(917)	69.2	(617)
- Pre-menopausal	-	-	-	-	-	-	31.1	(9,022)	31.5	(494)	17.7	(158)
Medical history (% (n))												
- Hypertension	14.5	(3,762)	14.9	(269)	22.1	(472)	17.2	(4,984)	17.3	(272)	31.1	(277)
- Hypercholesterolemia	7.7	(2,005)	8.4	(151)	11.8	(253)	6.1	(1,762)	6.3	(99)	13.1	(117)
- Diabetes mellitus	2.6	(677)	2.9	(52)	5.4	(115)	1.5	(435)	1.5	(23)	4.4	(39)
Dietary variables (g/day)												
- Fruit (excl. Juice)	117.7	(27.0;324.7)	120.7	(27.8;349.0)	111.0	(25.1;313.2)	172.4	(49.1;417.4)	173.3	(51.1;423.1)	160.6	(37.5;399.2)
- Vegetables (excl. potatoes)	151.8	(62.7;291.4)	152.0	(60.3;300.8)	138.8	(56.7;277.5)	171.9	(69.8;329.4)	175.7	(70.0;328.9)	149.2	(54.5;318.3)
- Alcohol	19.4	(3.6;62.6)	19.7	(3.3;62.0)	18.2	(2.5;62.7)	9.3	(1.0;34.5)	9.4	(1.2;34.9)	6.5	(0.5;31.9)
- Total energy intake (MJ/day)	9.9	(7.1;13.6)	9.9	(7.0;13.6)	9.8	(7.1;13.4)	8.0	(5.7;11.2)	8.1	(5.7;11.1)	7.9	(5.5;11.1)
- Fatty fish	15.1	(4.4;37.8)	14.9	(4.1;36.5)	14.9	(4.1;37.2)	12.2	(3.6;31.8)	11.9	(3.4;33.8)	11.6	(2.6;31.0)
- Lean fish	24.4	(10.1;50.0)	24.9	(9.9;50.5)	24.2	(10.4;51.2)	21.0	(9.2;42.6)	20.9	(9.5;42.4)	20.6	(8.5;43.3)
- Total marine n-3 PUFA	0.7	(0.3;1.4)	0.7	(0.3;1.4)	0.7	(0.3;1.4)	0.6	(0.2;1.1)	0.6	(0.2;1.2)	0.6	(0.2;1.1)

Continuous variables are reported as medians (10th and 90th percentiles). Categorical variables are reported as percentage (n).

5.1. STUDY I

Aim

In the first study we evaluated dietary intake of fish and the association with incident MI. Furthermore, fish consumption was sub-divided into fatty and lean fish, and intakes of total and individual marine n-3 PUFA were calculated. The main hypothesis was that fish intake would be inversely associated with MI, and this association would be stronger for fatty fish than for lean fish.

Key methods

The study was based on the Danish Diet, Cancer and Health study. Data for this study originated from detailed semi-quantitative food frequency questionnaires including 26 specific questions regarding fish intake. Fish consumption was divided into fatty or lean fish based on the content of n-3 PUFA (more or less than 1g n-3 PUFA per 100g of fish) in the specific fish species. Furthermore, intake of total and specific n-3 PUFA was calculated based on Danish food composition tables. Data were analysed in a traditional cohort design using Cox proportional multivariate hazards models to evaluate the association between various measures of fish intake and incident MI.

Main results

A total of 3,089 cases of incident MI were identified and validated during a median follow-up time of 17 years. Baseline characteristics are reported in Table 3, p34 and the cohort selection process is illustrated in Figure 5, p33. After exclusions for missing co-variates 3,028 cases were included in the analyses. Fatty fish was inversely associated with incident MI for both men and women when comparing the highest quintile with the lowest (Table 4). For men, the association was modest with a HR=0.88 (95% CI: 0.77;1.00), and more pronounced for women with a HR=0.78 (95% CI: 0.63;0.96) after adjustments for potential confounding. Lean fish was not associated with the risk of incident MI.

Limitations

First of all, the study was based on a prospective cohort design with a thorough case validation and almost no loss to follow-up. We considered a few limitations. A frequent concern about data based on questionnaires is the risk of measurement error and bias and though we consider the questionnaires to be of high quality, we cannot rule out the potential of bias. One very important issue was the relatively long follow-up during which the diet was not re-evaluated. Thus, it is possible that the dietary habits, recorded at baseline, might change over time as well as other lifestyle and demographic data. This could potentially bias our results and limit our ability to control confounding. However, our study population was 50-64 years of age at baseline and middle-aged and elderly people are probably less likely to change their diet and lifestyle than young people. Indeed, a study on the general population of Denmark, showed that fish consumption was rather constant during 1995-2004 (119).

Main conclusions

For both men and women, incident MI was inversely associated with dietary intake of fatty fish, while lean fish was not associated with MI.

Table 4. Association between dietary fish consumption and incident MI

Men								
	Model A1*		**Model A2****		**Model B*****		**Model C********	
Fatty fish								
Q1 (0-8 g)	1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Q2 (>8-13 g)	0.88	(0.77;1.00)	0.89	(0.79;1.03)	0.90	(0.79;1.03)	0.91	(0.80;1.04)
Q3 (>13-18 g)	0.84	(0.73;0.96)	0.89	(0.78;1.01)	0.89	(0.78;1.02)	0.90	(0.79;1.03)
Q4 (>18-28 g)	0.82	(0.72;0.94)	0.90	(0.79;1.03)	0.90	(0.78;1.03)	0.92	(0.80;1.06)
Q5 (>28 g)	0.83	(0.72;0.94)	0.91	(0.79;1.04)	0.88	(0.77;1.00)	0.93	(0.81;1.07)
Lean fish								
Q1 (0-14 g)	1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Q2 (>14-21 g)	1.07	(0.94;1.23)	1.12	(0.98;1.29)	1.13	(0.99;1.29)	1.14	(1.00;1.31)
Q3 (>21-28 g)	1.05	(0.92;1.21)	1.11	(0.97;1.27)	1.10	(0.96;1.26)	1.14	(0.99;1.30)
Q4 (>28-39 g)	1.01	(0.89;1.16)	1.09	(0.95;1.25)	1.07	(0.94;1.23)	1.12	(0.97;1.29)
Q5 (>39 g)	1.04	(0.91;1.19)	1.07	(0.93;1.23)	1.05	(0.91;1.20)	1.12	(0.97;1.29)
Women								
	Model A1*		**Model A2****		**Model B*****		**Model C********	
Fatty fish								
Q1 (0-6 g)	1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Q2 (>6-10 g)	0.86	(0.70;1.06)	0.95	(0.77;1.16)	0.95	(0.77;1.16)	0.96	(0.78;1.18)
Q3 (>10-15 g)	0.85	(0.69;1.04)	0.96	(0.78;1.17)	0.93	(0.76;1.15)	0.98	(0.80;1.21)
Q4 (>15-23 g)	0.90	(0.74;1.10)	1.07	(0.87;1.31)	1.04	(0.85;1.27)	1.11	(0.90;1.36)
Q5 (>23 g)	0.69	(0.56;0.85)	0.83	(0.67;1.03)	0.78	(0.63;0.96)	0.86	(0.69;1.08)
Lean fish								
Q1 (0-13 g)	1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Q2 (>13-18 g)	0.94	(0.77;1.16)	1.00	(0.82;1.23)	1.01	(0.82;1.24)	1.03	(0.84;1.27)
Q3 (>18-24 g)	0.87	(0.71;1.07)	0.95	(0.77;1.17)	0.93	(0.76;1.15)	0.98	(0.79;1.21)
Q4 (>24-33 g)	0.90	(0.73;1.10)	0.99	(0.81;1.22)	0.98	(0.80;1.21)	1.04	(0.84;1.29)
Q5 (>33 g)	0.89	(0.72;1.09)	0.95	(0.77;1.16)	0.93	(0.75;1.14)	0.99	(0.79;1.24)

Cox proportional hazard models for men and women separately, reported as hazard ratios with 95% confidence intervals in parentheses.
*Model A1: Crude analysis.
**Model A2: Adjusted for traditional risk factors including: Smoking, body mass index, waist circumference, physical activity, alcohol intake, educational level, menopausal status(women).
***Model B: Adjusted as model A2 with additional covariates: History of diabetes mellitus, hypertension and hypercholesterolaemia.
****Model C: Adjusted as model A2 with additional dietary covariates: Total energy intake, intake of fruits and vegetables and intake of nuts.

5.2. STUDY II

Aim

In this study, we investigated the association between the content of total and individual marine n-3 PUFA in adipose tissue and incident MI. We hypothesised that total and individual n-3 PUFA would be inversely associated with incident MI.

Key methods

Using a case-cohort design, a randomly selected sub-cohort (n=3,500) was drawn within the Danish Diet, Cancer and Health study, to represent the entire cohort. Thus, the study cohort included cases of incident MI and the sub-cohort. Adipose tissue samples were collected at baseline and their composition was analysed by gas chromatography. Statistical analyses were performed using weighted Cox proportional multivariate hazard models.

Main results

Baseline characteristics are reported in Table 3, p34 and the cohort selection process is illustrated in Figure 5, p33. Notably, 421 subjects had missing adipose tissue biopsies and a total of 2,814 cases were included in the analyses. We examined the association of the three major marine n-3 PUFA (EPA, DPA and DHA) with incident MI (Table 5). In both men and women we found an inverse relationship between the content of EPA in adipose tissue and MI when comparing the highest and lowest exposure quintile. The association did not change substantially after multivariate adjustment and remained statistically significant with a HR of 0.76 (95% CI: 0.60;0.97) for men and a HR of 0.70 (95% CI: 0.49;0.99) for women when adjustments were applied (model B). For men, the same tendency was observed for DHA with a negative association in the fourth and fifth quintile. However, multivariate adjustment for traditional risk factors for MI attenuated the association, and adjusted analyses were borderline significant. In women we found no consistent association between DHA content and MI. Further, no consistent associations between DPA content and MI were detected for neither men nor women. In addition to the analyses of quintiles, adipose tissue n-3 PUFA were evaluated continuously as restricted cubic splines with three knots (Figure 6) indicating an inverse association between EPA and MI, as observed for the quintiles.

Limitations

This study used a nested case-cohort design, which implies that a sub-sample of the whole cohort was drawn to represent the cohort. Thus, the sample size for this study was smaller than for Study I. However, this issue was addressed by assigning weights to each non-case from the sub-cohort and implementing this weighting scheme in the Cox regression. Furthermore, the sub-cohort resembled the cohort on all baseline parameters (Table 3, p34). Compared with questionnaires, adipose tissue fatty acids have the advantage, as a biomarker, of being an objective measure of the exposure,

and as such less prone to bias. Importantly, it was evident from correlations between dietary intake of n-3 PUFA and adipose tissue content (Table 6), that adipose tissue was only moderately correlated with dietary intake, and adipose tissue n-3 PUFA may more truly reflect the endogenous exposure to these fatty acids than their dietary intake. Furthermore, we used the relative proportion of fatty acids and not their absolute concentration, which is conventional but might be considered a weakness.

Main conclusions

The content of EPA was inversely associated with incident MI in both men and women, and while the same tendency was observed for DHA, associations were only borderline significant. DPA was not associated with incident MI.

Table 5. Association between the adipose tissue content of marine n-3 PUFA and incident MI.

Men								
	Model A1*		**Model A2****		**Model B*****		**Model C********	
Content of EPA (%)								
Q1 (0.00-0.07)	1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Q2 (>0.07-0.09)	1.01	(0.83;1.23)	0.99	(0.80;1.23)	1.00	(0.80;1.24)	0.96	(0.77;1.20)
Q3 (>0.09-0.11)	0.83	(0.68;1.02)	0.80	(0.64;1.01)	0.82	(0.65;1.04)	0.77	(0.60;0.98)
Q4 (>0.11-0.14)	0.93	(0.76;1.14)	0.96	(0.77;1.19)	0.92	(0.74;1.16)	0.86	(0.66;1.10)
Q5 (>0.14)	0.74	(0.60;0.92)	0.79	(0.62;1.00)	0.76	(0.60;0.97)	0.65	(0.47;0.90)
Content of DPA (%)								
Q1 (0.00-0.21)	1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Q2 (>0.21-0.24)	1.21	(0.99;1.49)	1.21	(0.98;1.51)	1.22	(0.98;1.53)	1.26	(1.00;1.57)
Q3 (>0.24-0.28)	1.10	(0.89;1.35)	1.16	(0.93;1.44)	1.16	(0.92;1.45)	1.19	(0.93;1.52)
Q4 (>0.28-0.33)	1.09	(0.89;1.34)	1.12	(0.89;1.41)	1.08	(0.86;1.37)	1.17	(0.90;1.52)
Q5 (>0.33)	1.03	(0.84;1.27)	1.09	(0.87;1.38)	1.00	(0.79;1.27)	1.14	(0.84;1.54)
Content of DHA (%)								
Q1 (0.00-0.17)	1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Q2 (>0.17-0.22)	1.00	(0.82;1.23)	0.95	(0.77;1.18)	0.92	(0.74;1.15)	0.95	(0.76;1.20)
Q3 (>0.22-0.27)	0.96	(0.78;1.18)	0.99	(0.79;1.23)	0.95	(0.76;1.19)	0.95	(0.74;1.21)
Q4 (>0.27-0.35)	0.80	(0.65;0.99)	0.88	(0.71;1.10)	0.88	(0.70;1.10)	0.85	(0.63;1.06)
Q5 (>0.35)	0.79	(0.64;0.97)	0.88	(0.70;1.10)	0.81	(0.64;1.01)	0.77	(0.56;1.06)
Women								
	Model A1*		**Model A2****		**Model B*****		**Model C********	
Content of EPA (%)								
Q1 (0.00-0.06)	1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Q2 (>0.06-0.08)	1.00	(0.76;1.31)	0.97	(0.71;1.31)	0.97	(0.71;1.32)	0.93	(0.68;1.28)
Q3 (>0.08-0.10)	1.11	(0.83;1.49)	1.14	(0.82;1.58)	1.10	(0.78;1.54)	1.06	(0.74;1.50)
Q4 (>0.10-0.13)	0.96	(0.74;1.25)	1.00	(0.74;1.35)	0.99	(0.72;1.34)	0.83	(0.58;1.19)
Q5 (>0.13)	0.69	(0.52;0.92)	0.80	(0.57;1.11)	0.70	(0.49;0.99)	0.59	(0.37;0.92)
Content of DPA (%)								
Q1 (0.00-0.22)	1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Q2 (>0.22-0.26)	1.18	(0.89;1.57)	1.19	(0.86;1.64)	1.15	(0.83;1.60)	1.24	(0.89;1.73)
Q3 (>0.26-0.31)	1.12	(0.85;1.49)	1.09	(0.78;1.51)	1.07	(0.76;1.50)	1.16	(0.79;1.68)
Q4 (>0.31-0.36)	1.35	(1.02;1.80)	1.41	(1.00;1.97)	1.35	(0.96;1.89)	1.45	(0.98;2.14)
Q5 (>0.36)	1.17	(0.88;1.54)	1.16	(0.83;1.63)	1.05	(0.74;1.48)	1.15	(0.73;1.82)
Content of DHA (%)								
Q1 (0.00-0.19)	1 (ref)		1 (ref)		1 (ref)		1 (ref)	

Q2 (>0.19-0.25)	0.78	(0.59;1.03)	0.76	(0.56;1.04)	0.77	(0.56;1.05)	0.71	(0.52;0.98)
Q3 (>0.25-0.30)	0.91	(0.69;1.21)	0.96	(0.69;1.33)	0.97	(0.69;1.36)	0.90	(0.63;1.30)
Q4 (>0.30-0.39)	1.05	(0.80;1.38)	1.11	(0.81;1.53)	1.10	(0.80;1.52)	0.97	(0.66;1.41)
Q5 (>0.39)	0.68	(0.51;0.91)	0.88	(0.63;1.22)	0.81	(0.57;1.15)	0.74	(0.46;1.20)

Abbreviations: EPA, Eicosapentaenoic acid; DPA, Docosapentaenoic acid; DHA, Docosahexaenoic acid.
Cox proportional hazard models for men and women separately, reported as hazard ratios with 95% confidence intervals in parenthesis.
*Model A1: Crude analysis.
**Model A2: Adjusted for traditional risk factors including: Smoking, body mass index, waist circumference, physical activity, alcohol intake, educational level, menopausal status(women).
***Model B: Adjusted as model A2 with additional covariates: History of diabetes mellitus, hypertension and hypercholesterolaemia.
****Model C: Adjusted as model A2 with additional covariates: Adipose tissue content of total saturated, monounsaturated and polyunsaturated (excl. marine n-3 PUFA) fatty acids and dietary fiber.

Table 6. Correlations between dietary intake and adipose tissue n-3 PUFA.

Men						
	Adipose tissue			**Dietary intake**		
	EPA	DPA	DHA	EPA	DPA	DHA
Adipose tissue						
EPA	1					
DPA	0.74	1				
DHA	0.86	0.82	1			
Dietary intake						
EPA	**0.37**	0.29	0.41	1		
DPA	0.17	**0.15**	0.21	0.77	1	
DHA	0.33	0.26	**0.37**	0.97	0.82	1
Women						
	Adipose tissue			**Dietary intake**		
	EPA	DPA	DHA	EPA	DPA	DHA
Adipose tissue						
EPA	1					
DPA	0.74	1				
DHA	0.84	0.79	1			
Dietary intake						
EPA	**0.33**	0.23	0.37	1		
DPA	0.17	**0.11**	0.21	0.76	1	
DHA	0.30	0.20	**0.34**	0.97	0.83	1

Abbreviations: EPA, Eicosapentaenoic acid; DPA, Docosapentaenoic acid; DHA, Docosahexaenoic acid.
Pearson's correlation coefficients presented separately for men and women. Correlations between adipose tissue n-3 fatty acids and their corresponding dietary n-3 fatty acids are marked by bold figures.

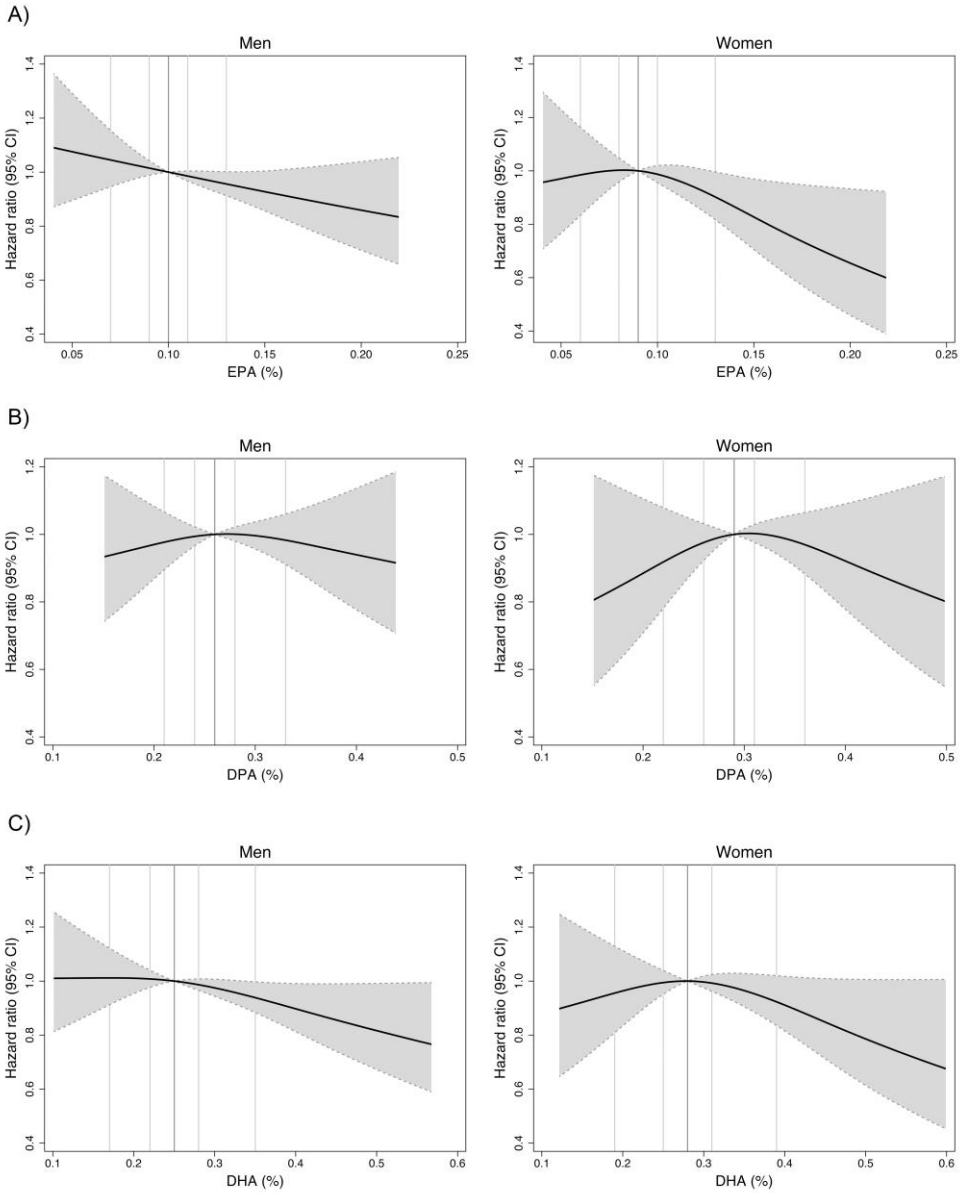

Figure 6. Spline curves illustrating the association between the adipose tissue content of specific n-3 PUFA and incident MI. Restricted cubic spline curves with three knots showing hazard ratios with 95% confidence interval (grey area) for the association between incident myocardial infarction (MI) and adipose tissue content of (A) EPA, (B) DPA and (C) DHA. Results are presented for men and women separately with the median content as reference. Vertical lines indicate quintiles of fatty acid content.

5.3. STUDY III

Aim

In the third study we investigated four candidate genes in the 5-LOX pathway. Our aim was to examine associations between individual polymorphisms and inferred haplotypes with incident MI. The main hypothesis was that genetic variants would be associated with MI.

Key methods

In a nested case-cohort study design based on the Diet Cancer and Health study, we conducted a genetic association study examining four candidate genes in the 5-LOX pathway (*ALOX-5, FLAP, LTA4-H* and *LTC4-S*). A randomly selected sub-cohort (n=3,000) was drawn to represent the cohort. We selected 22 SNPs and a tandem-repeat polymorphism primarily based on previous association with atherosclerotic disease. SNPs were genotyped by LGC Genomics, UK, using the KASP^{TM} genotyping assay, while the tandem repeat was genotyped by sequencing technique. Measures of association were assessed by Cox proportional hazard models using a weighting scheme to account for the size of the nested sub-cohort. Haplotypes were inferred using PHASE 2.1, and weights were assigned to each subject based on the probability of each pair of haplotypes derived from PHASE.

Main results

In total, 2,876 cases were included in the analyses after exclusions (Figure 5, p33). Analysing the tandem repeat polymorphism in *ALOX-5* (rs59439148) we found a positive association with MI in men, while no association was detected in women. The pooled estimates showed the same pattern as for men (Table 7). A number of SNPs were associated with incident MI when comparing homozygotes carriers of the minor allele with homozygotes for the major allele. In *FLAP* we found two markers (rs9551963 and rs17222842) that were negatively associated with MI, while in *LTA4-H,* rs2247570 was positively associated with MI when comparing homozygotes for the minor allele with homozygotes for the major allele (Table 7). Previously reported haplotypes were not associated with MI in our population.

Limitations

The present study was designed as a candidate gene study which has the clear advantage of providing a high power to detect genotype-phenotype associations with a relatively limited sample size compared with genome wide association studies. Our study were limited by only investigating the four candidate genes selected for the study, while there might be other genes involved in regulating the 5-LOX pathway. A frequent problem in genetic association studies is the number of statistical tests performed, which may increase the chance of type I errors. This was addressed by limiting the number of SNP markers for the current study, by testing only one genetic model of inheritance and testing only one crude and one adjusted model.

Main conclusions

A number of individual SNPs and the tandem repeat polymorphism were associated with incident MI. However, associations were modest and haplotype associations did not reveal any clear associations with the phenotype. Collectively, the study suggests that the 5-LOX pathway may play a role for the risk of incident MI.

Table 7. Polymorphisms associated with incident MI.

SNP	Genotype	Model A1*		Model A2**	
ALOX-5					
rs59439148	W/W	1 (ref)		1 (ref)	
	W/V	0.94	(0.83;1.06)	0.96	(0.84;1.10)
	V/V	1.23	(0.89;1.71)	1.35	(0.96;1.90)
FLAP					
rs9551963	C/C	1 (ref)		1 (ref)	
	C/A	0.86	(0.75;0.98)[a]	0.83	(0.72;0.96)[a]
	A/A	0.85	(0.73;0.99)[a]	0.80	(0.68;0.95)[a]
rs17222842	G/G	1 (ref)		1 (ref)	
	G/A	0.97	(0.85;1.12)	0.94	(0.81;1.10)
	A/A	0.40	(0.22;0.73)[a]	0.44	(0.24;0.82)[a]
LTA4-H					
rs2247570	A/A	1 (ref)		1 (ref)	
	A/G	1.00	(0.90;1.13)	1.00	(0.88;1.13)
	G/G	1.23	(1.01;1.51)[a]	1.28	(1.03;1.59)[a]

Abbreviations: SNP, Single nucleotide polymorphism; ALOX-5, Arachidonate 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; LTC4-S, Leukotriene C4 synthase; LTA4-H, Leukotriene A4 hydroxylase.
The table displays hazard ratios from weighted Cox proportional hazard models. Alleles correspond to the positive DNA-strand according to dbSNP, human assembly GRCh38.p2.
*Model A1: Crude analyses adjusted for sex.
**Model A2: Adjusted analyses including sex, smoking status, educational level, physical activity, BMI, waist circumferrence and alcohol consumption.
[a]*p*-value < 0.05, not adjusted for multiple comparisons.

5.4. STUDY IV

Aim

This study investigated possible interaction between substrates for the ALOX-5 enzyme (EPA and AA) and the tandem repeat polymorphism, located in the promotor region of the *ALOX-5* gene, in relation to incident MI. We hypothesised that the effect of the polymorphism would be modulated by the content of EPA and AA in adipose tissue, where EPA would blunt the effect of the variant genotype while AA would augment the effect.

Key methods

To evaluate interaction between genotype and ALOX-5 substrates we used data from DNA samples and adipose tissue biopsies. The tandem polymorphism was genotyped by multi-titre plate sequencing determining the number of tandem repeats for each

allele and adipose tissue content was determined by gas chromatography. A case-cohort design was used to analyse data. Measures of association were assessed using Cox proportional hazards models. Potential interaction between genotype and adipose tissue EPA or AA was investigated by cross-tabulating HRs for genotype by quintiles of EPA or AA. Furthermore, we calculated the relative excess risk due to interaction (RERI) to determine biological interaction quantitatively.

Main results

The study included 2,680 cases after exclusion of participants with missing data on adipose tissue biopsies and/or genotype information (Figure 5, p33). Overall, the results indicated a higher risk of MI for homozygous carriers of the variant alleles compared to carriers of the wildtype. When cross-tabulating the genotype by quintiles of EPA or AA content in adipose tissue (Table 8), we found the highest risk for MI when comparing the lowest quintile group of EPA for homozygous carriers of the variants with the reference group (HR=2.15, 95% CI: 0.91;5.09), while for AA, the highest risk of MI was found when comparing the highest quintile stratum of AA content for homozygotes for the variants compared to the reference (HR=3.02, 95% CI: 1.41;6;44). RERI were calculated to assess interaction on an additive scale, and measures of association were not statistically significant.

Limitations

Despite a relatively large number of cases and sub-cohort members that had information on both genotype and adipose tissue fatty acids, the number of homozygous carriers was limited. This made assessments for interaction challenging, when cross-tabulating genotype by quintiles of EPA and AA content. Furthermore, sex-stratified analyses did not yield meaningful results for women because of the limited number of female cases. This raises the concern that the study was underpowered to detect possible interaction by adipose tissue EPA and AA.

Main conclusions

Results indicated that associations between genotype and incident MI were modulated by EPA and AA, where EPA attenuated the effect and AA exacerbated the effect, albeit analyses of interaction by RERI were not statistically significant.

Table 8. Cross-tabulation of genotype by quintiles of EPA and AA. Hazard ratios for the association with incident MI.

	Genotype															
	Model A1*				***Model A2*****				***Model B*****				***Model C******			
EPA	**W/W, W/V**		**V/V**		**W/W, W/V**		**V/V**		**W/W, W/V**		**V/V**		**W/W, W/V**		**V/V**	
Q5	1 (ref)		0.85	(0.39;1.82)	1 (ref)		0.89	(0.39;2.02)	1 (ref)		1.16	(0.52;2.59)	1 (ref)		0.85	(0.37;1.96)
Q4	1.18	(0.98;1.41)	1.02	(0.50;2.06)	1.12	(0.92;1.35)	1.20	(0.60;2.41)	1.17	(0.96;1.43)	1.31	(0.63;2.74)	1.17	(0.96;1.43)	1.32	(0.66;2.65)
Q3	1.09	(0.90;1.32)	1.50	(0.53;4.26)	0.95	(0.77;1.17)	1.59	(0.61;4.13)	1.03	(0.83;1.28)	1.62	(0.59;4.46)	1.04	(0.83;1.29)	1.61	(0.61;4.23)
Q2	1.26	(1.06;1.51)	1.88	(1.00;3.53)	1.12	(0.92;1.36)	1.83	(0.98;3.43)	1.24	(1.01;1.52)	1.82	(0.90;3.65)	1.23	(1.00;1.51)	2.03	(1.08;3.84)
Q1	1.30	(1.09;1.55)	2.31	(1.08;4.96)	1.18	(0.97;1.43)	1.70	(0.69;4.15)	1.29	(1.05;1.58)	2.15	(0.91;5.09)	1.31	(1.06;1.62)	1.92	(0.76:4.83)
AA																
Q1	1 (ref)		1.48	(0.69;3.16)	1 (ref)		1.37	(0.61;3.07)	1 (ref)		1.26	(0.52;3.07)	1 (ref)		1.37	(0.60;3.12)
Q2	1.08	(0.90;1.29)	1.30	(0.65;2.60)	1.07	(0.88;1.29)	1.29	(0.60;2.79)	1.05	(0.87;1.27)	1.37	(0.66;2.86)	1.09	(0.89;1.32)	1.31	(0.61;2.86)
Q3	1.26	(1.05;1.50)	1.18	(0.56;2.49)	1.18	(0.97;1.43)	1.15	(0.55;2.39)	1.16	(0.95;1.42)	1.15	(0.55;2.42)	1.20	(0.98;1.46)	1.11	(0.53;2.35)
Q4	1.46	(1.21;1.75)	1.39	(0.64;3.02)	1.24	(1.01;1.52)	1.60	(0.71;3.60)	1.16	(0.93;1.43)	1.50	(0.65;3.50)	1.24	(1.00;1.53)	1.66	(0.74;3.72)
Q5	1.61	(1.35;1.93)	2.91	(1.40;6.03)	1.34	(1.09;1.66)	2.57	(1.19;5.54)	1.17	(0.94;1.46)	3.02	(1.41;6.44)	1.32	(1.05;1.65)	2.50	(1.14;5.52)

Abbreviations: EPA, eicospentaenoic acid; AA, arachidonic acid; W, wildtype allele; V, variant allele.
Hazard ratios with 95 % confidence intervals in (parentheses) from Cox proportional hazards model cross tabulated by quintiles of EPA or AA in adipose tissue and genotype of rs59439148. The reference group was the lowest quintile of fatty acids and carriers of one or two wildtype alleles.
*Model A1: Crude analyses adjusted for sex.
**Model A2: Adjusted for lifestyle and demographic measures, including sex, smoking status, educational level, physical activity, BMI, waist circumference and alcohol consumption.
***Model B: Adjusted as model A2 with additional medical history variables, including History of diabetes mellitus, hypertension and hypercholesterolaemia.
****Model C: Adjusted as model A2 with additional dietary variables, including adipose tissue content of total saturated, monounsaturated and trans fatty acids and dietary fiber.

CHAPTER 6. DISCUSSION

The overall aim of the thesis was to investigate whether fish consumption and marine n-3 PUFA were associated with incident MI. Furthermore, genetic variants in the 5-LOX pathway was examined and the interaction between a functional polymorphism in the ALOX-5 gene and fatty acids substrates for the pathway was explored. We investigated the dietary consumption of fish based on food frequency questionnaires (Study I), and next, the adipose tissue content of major marine n-3 PUFA was evaluated (Study II). The studies supported an inverse association with MI for fatty fish and adipose tissue content of EPA. Next, we conducted a candidate gene study, exploring genetic variants in the 5-LOX pathway (Study III). Individual SNPs were associated with MI, but in general associations were modest, and previously reported haplotypes were not associated with MI. In Study IV, a functional tandem repeat in *ALOX-5* was studied, examining interaction between the polymorphism and adipose tissue content of EPA or AA (substrates for the 5-LOX pathway). The data indicated that the content of EPA and AA modified the risk imposed by the variant genotype. However, quantitative measures of interaction were, not statistically significant.

Evaluating dietary fish intake – biomarkers vs. dietary questionnaires

The studies included in this thesis were observational studies based on the Danish prospective cohort study, the Diet, Cancer and Health Study. Essential issues in observational studies relate to how outcome is assessed, which was explained in detail earlier, "Chapter 4. Methods". Importantly, Joensen et al. (113) validated diagnoses of MI from national registries through 2003, and an algorithm was established to validate new diagnoses from January 2004 and forth, which was used in the present thesis.

Another important issue was how to evaluate the exposure. The intake of marine n-3 PUFA was evaluated by two different approaches. First, fish intake was assessed using semi-quantitative food frequency questionnaires, and secondly, adipose tissue content of marine n-3 PUFA, a long-term biomarker of the dietary intake, was determined.

The dietary questionnaire was detailed, including 26 specific questions concerning intake of fish and food products containing fish, thereby allowing us to differentiate between types of fish and to categorise fish intake according to the relative amount of n-3 PUFA. Furthermore, the questionnaire had been carefully developed and validated by comparison with two times seven days weighed dietary recordings (111,120). However, some concerns remain. First of all, it may be difficult for subjects to recall and make an accurate assessment of their average food intake. This becomes even more difficult as the period of recall becomes longer, and periodical variations in food consumption are likely to be reflected in the dietary questionnaire

although participants were asked to report their average intake of the food items during the last 12 months. Apart from this general issue, a number of innate problems may be related to dietary assessment from questionnaires, including reporting of complex food items, errors in calculating more specific food items from food composition tables, systematic over- / under-reporting of specific food items and errors in estimating correct portion sizes. Importantly, the questionnaire was filled-in at baseline with no specific focus on the exposure and before participants had any knowledge about future disease. Thus, potential measurement error including information bias was unlikely to be differentiated, and possible random error caused by imprecise estimation of the individual food intake would be, at least partly, compensated by the size of the cohort.

Biomarkers of n-3 PUFA intake represents an objective measure whereas dietary recordings are prone to subjectivity, and specific fatty acids can be determined with high precision. We used adipose tissue content of marine n-3 PUFA as an objective measure of n-3 PUFA, which is a widely used biomarker, reflecting long-term exposure to these fatty acids (21). Thus, based on the long half-life of white adipose tissue cells of approximately 600 days, it has been suggested that adipose tissue may reflect the average dietary intake during the last 1-3 years (121). However, the fatty acid composition of adipose tissue is dependent, not only on the dietary intake, but also on the uptake and release from adipose tissue as well as their metabolism. Notably, marine n-3 PUFA can be synthesised endogenously from the conversion of ALA, although only to a limited extent (12,122). However, considering that the dietary intake of ALA often is considerably higher than the intake of marine n-3 PUFA, the amount of long-chained n-3 PUFA derived from ALA might not be trivial. Therefore, adipose tissue content may more truly reflect the endogenous exposure to n-3 PUFA and, as a consequence of the dependency on endogenous processes, may reflect the dietary intake less precisely. Indeed, the correlations between dietary intake and adipose tissue content of n-3 PUFA were modest (Table 6, p39). However, the endogenous exposure may be a valuable measure of exposure compared to the true dietary exposure, depending on the specific questions asked (123–125). Taken together, dietary questionnaires and adipose tissue biopsies have their individual advantages and disadvantages, and as such, they should be regarded as complementary in evaluating the true exposure to n-3 PUFA.

Association of fish consumption and adipose tissue content of marine n-3 PUFA with incident MI

The main findings from Study I & II were that dietary intake of fatty fish was inversely related to incident MI. In contrast, no association could be demonstrated between lean fish intake and MI. Furthermore, the content of EPA in adipose tissue was negatively associated with incident MI. The same tendency was observed for DHA (non-significant), whereas DPA was not associated with incident MI.

In the Diet, Cancer and Health cohort, two earlier studies had been conducted examining the association of fish intake and adipose tissue marine n-3 PUFA content in participants with ACS (35,36). Thus, Bjerregaard et al. (36) found an inverse relationship between intake of fatty fish and ACS in men, suggesting a HR of 0.68 from eating a moderate to high amount of fatty fish. Another study on adipose tissue n-3 PUFA demonstrated a lower risk of ACS in men with a high content of marine n-3 PUFA (35). Findings in women were non-significant without a concise pattern for both studies. In Study I we also demonstrated a negative association in men, but the associations were somewhat attenuated during follow-up, and our results suggested a modest HR of 0.88, when comparing the lowest and the highest quintile in men. In light of these inconsistencies, we performed additional analyses dividing the study period according to the earlier studies and found the same associations as previously observed. However, it is not unusual to observe a weakening of the associations in cohort studies during long follow-up periods, which may be explained by a decline in the preventive potential of the exposure, simply because the subjects that had a high effect of n-3 PUFA intake are the same subjects that have a high risk of experiencing an event (MI). Furthermore, as a result of higher age among the participants during longer follow-up, the relative risk ratio for experiencing an MI may decrease between the group with high fish intake and low fish intake because age becomes a more dominant risk factor. Other explanations, such as cohort-wide changes in lifestyle and standard medical treatment towards a more heart healthy profile, may help explain our findings as well. In women, Study I & II showed a more consistent pattern compared to the earlier studies and suggested a HR of 0.78 when comparing the highest quintile of fatty fish with the lowest. Our studies included a considerably larger number of cases (*n*=3,089) compared to the previous dietary study (*n*=1,122), and particularly with regard to the analyses on adipose tissue biopsies (*n*=1,012). Notably, the number of female cases was larger providing considerably more strength to explore the associations in both men and women.

Epidemiological studies have generally provided evidence for a protective role of fish consumption (31–38,40), supported by secondary intervention trials (42,43,126) associating fish intake and fish oil supplements with a lower risk of CHD and sudden cardiac death. Summarising data from prospective cohort studies, He et al. conducted a meta-analysis in 2004, suggesting that 1 fish serving/week was associated with a 15% lower risk of fatal CHD, and 2-4 servings/week with 23% lower risk. These earlier trials lead to the conclusion among many researchers and scientific communities that intake of fish and fish oil supplements reduces the risk of CHD. Consequently, both the European Society of Cardiology (46) and the American Heart Association (45) recommend a daily intake of marine n-3 PUFA of 1 g/day for the secondary prevention of CHD. However, more recent trials have come to question the beneficial effects from dietary n-3 PUFA supplements on CHD (47–49). Several reasons might explain these findings (127). First of all, methodological variations, different populations and different interventions might explain some of the differences. Notably, recent trials were all conducted in populations with aggressive background medical therapy,

including lipid lowering, anti-hypertensive and anti-platelet therapy. In support of this notion, post hoc analyses from the Alpha Omega Trial found no effect of n-3 PUFA supplements in statin users, but in contrast, n-3 PUFA supplementation was associated with a 50% reduction in major CVD events among non-statin users (128). Further, the beneficial effect of n-3 PUFA in earlier trials was mainly reported for fatal CHD, while recent trials have focused on non-fatal or composite endpoints for CHD. Finally, the background dietary fish intake was generally higher in recent trials in accordance with rising public awareness of a healthy lifestyle.

In line with this, a recent meta-analysis (51) of secondary prevention trials reported a modest effect of marine n-3 PUFA on cardiac death (RR=0.91), sudden death (RR=0.87) and MI (RR=0.89). Moreover, associations were borderline significant, and the authors concluded that there was insufficient support for recommending fish oil supplements and fish intake for the secondary prevention of CVD. Mozaffarian et al. (52) also reviewed the evidence for n-3 PUFA and presented a combined meta-analysis, including both observational studies and randomised controlled trials, suggesting a beneficial effect of n-3 PUFA intake on various CVD endpoints. Most recently, another meta-analysis presented pooled estimates from 19 cohort studies using a variety of biomarkers for n-3 PUFA and found a modest relative risk reduction of fatal CHD events of around 9% for various n-3 PUFA (53). Taken together, recent meta-analyses have reported a more modest effect of marine n-3 PUFA in the prevention of CVD (52,53), and while individual differences between the meta-analyses may be explained by the type of trials included, different endpoints among other differences in study design, there remains a discrepancy between most of the earlier trials and more recent trials examining n-3 PUFA and CVD. Where earlier trials, more convincingly, reported a beneficial effect on the prevention of CVD, and CHD in particular, some of the more recent studies have been inconclusive or neutral. The recent studies have stimulated renewed interest in n-3 PUFA, and dietary recommendations regarding fish consumption and fish oil supplementation in CVD prevention are currently being debated.

Association of genetic polymorphisms in the 5-LOX pathway with incident MI

To explore associations between genetic polymorphisms in the 5-LOX pathway and MI we conducted a candidate gene study examining the four genes encoding key enzymes in the pathway (Study III). A number of individual SNPs were associated with MI. However, associations were modest, and most of the associations were not statistically significant when applying corrections for multiple comparisons.

A number of earlier genetic association studies have examined the association between polymorphisms in the 5-LOX pathway and various cardiovascular endpoints including ischemic stroke and CHD, as earlier reviewed. The following is a discussion of the evidence for each candidate gene and our results.

In *ALOX-5* the tandem repeat polymorphism, rs59439148, was first found to be associated with higher intima-media thickness (88), a marker of atherosclerosis, but replication studies on cases with CHD, have been conflicting. Importantly, two independent studies in MI patients of Northern European origin (genetically close related to our population) did not find any association between variant alleles and MI (90,91). Another study, including a mixed population of Caucasians and African-Americans, reported a positive association in African-Americans but not in Caucasians (92). In Study III, we found a positive association between homozygous carriers of the variant alleles and MI, but the association was only significant in men, while there was no association among women. Other SNPs have been explored in *ALOX-5* (92,94), but only rs59439148 and SNPs linked to this polymorphism have been associated with atherosclerosis and atherothrombotic disease.

In *FLAP*, the deCODE investigators identified two haplotypes (Hap-A and Hap-B), that were associated with risk of MI and/or ischemic stroke in three independent populations of Caucasians (95,96). Although, we could not confirm the association between previously defined haplotypes and MI in our population, we found two individual SNPs within the haplotype blocks that were associated with MI when comparing homozygous carriers of the minor allele with homozygotes for the major allele (rs9551963 and rs17222842). Other studies have investigated the SNPs and haplotypes within FLAP, but again results have been conflicting (92,94,98–104), and no consensus has been established in favour of a certain SNP or possible functional polymorphism.

In *LTA4-H*, we analysed ten SNPs covering a haplotype block as previously described by the deCODE investigators (97). As for FLAP, we found some of the individual SNPs to be associated with incident MI, but the previously reported haplotype (Hap-K) was not associated with MI in our population.

To further investigate haplotype associations, and potentially identify new and unique haplotypes for our cohort, we performed a global haplotype analysis for *FLAP* and *LTA4-H* SNPs. In these analyses, we included all common haplotypes (minor allele frequency > 0.01) inferred to our cohort in a multivariate model using the most common haplotype as the reference haplotype. By this method we found sporadic associations between haplotypes and incident MI, but the associations were modest.

In conclusion, genetic association studies provide some evidence of a link between the 5-LOX pathway and atherosclerotic disease, which is supported by Study III. However, despite a number of studies performed, it has not been possible to consistently replicate initial findings and confirm the associations of specific polymorphism in most of the candidate genes. Genetic association studies are highly dependent on functional studies to establish causality and elucidate the biological significance of a polymorphism. In this respect, rs59439148 is the best documented polymorphism included in our study (76,89,129). Apart from this, Hap-A was

associated with a higher production of 4-series leukotrienes (95), indicating enhanced enzymatic activity from carrying this haplotype. Interestingly, several large genome wide association studies have been conducted since the deCODE studies, and none of these have pointed towards the 5-LOX pathway as being of major importance in CHD (130,131). These inconsistencies have led some authors to investigated the possibility that substrates of the 5-LOX pathway might interact with genetic variants in a complex environment where the effect of the genetic variant is dependent on substrate availability (88,89,105,106). Other authors have suggested that a "pathway approach" may be more appropriate, taking into account that each step of the 5-LOX pathway may influence the outcome all together. For instance, Crosslin et al. (104) examined how the expression of 5-LOX genes depended on the genotype of rs10507391, while another study found significant gene-gene interaction between a selected SNP from each of three 5-LOX genes (109). Aspects of gene-gene interactions were not explored during the work of this thesis.

Refining the question – diet X gene interaction

In Study IV we examined possible interaction between ALOX-5 substrates and the tandem repeat rs59439148. Cross-tabulation of genotype by quintiles of EPA or AA content indicated a higher risk of MI for carriers of two variant alleles as reported in Study III. While the association between genotype and MI was augmented by a higher content of AA, the opposite was observed for EPA, although interaction was not statistically significant when assessed quantitatively by calculating RERI.

Dwyer et. al (88), were the first to associate the *ALOX-5* tandem polymorphism with atherosclerosis traits, and also the first group to suggest interaction between dietary intake of 5-LOX substrates and the polymorphism. They found AA and marine n-3 PUFA to modulate the association of variant genotypes with carotid intima-media thickness in opposite directions, which was in line with our findings. Later, the same group performed a larger study on Costa Ricans, including 1885 cases of MI (89). While the study showed no overall association of the variant genotype, subjects with a high intake of AA had a significantly higher risk of MI when the variant genotype was present indicating interaction between AA intake and genotype. In contrast, no interaction was detected for marine n-3 PUFA. Both studies used dietary questionnaires to assess the intake of fatty acids, while our study used adipose tissue content as a biomarker reflecting long-term dietary intake and more precisely the endogenous exposure to the respective fatty acids.

Taken together, Study IV, along with other studies suggests that the association between the *ALOX-5* tandem repeat polymorphism and atherothrombotic disease is modified by substrate availability, and this may be an important aspect to consider when evaluating the association between genetic variants in the 5-LOX pathway and multifactorial disorders. During the work of this thesis, we did not explore diet-gene interaction in other candidate genes from the 5-LOX pathway, but interestingly, Zhao et al recently reported significant diet-gene interaction between a haplotype in *LTA4-*

H (Hap-E) and dietary intake of n-3 and n-6 PUFA (105,106). Therefore, it might be relevant to look for diet-gene interaction in other candidate genes of the 5-LOX pathway, which may help explain the heterogeneous results obtained from genetic association studies.

Mechanistic evidence linking marine n-3 PUFA and the 5-LOX pathway to atherothrombotic disease

The current thesis is based on prospective cohort studies, and this is important to keep in mind when discussing mechanistic aspects. Thus, the mechanisms by which n-3 PUFA influence the risk of MI in Study I & II are speculative.

Seafood may contain several components apart from n-3 PUFA, e.g. selenium, vitamin D, iodine and peptides, which might contribute to possible health benefits including possible effect on CVD (132). However, Study I suggested that the content of n-3 PUFA is important since only fatty fish and not lean or total fish intake was associated with incident MI. Furthermore, we examined individual marine n-3 PUFA in Study II, and these analyses suggested that EPA might play a more important role than DHA and DPA. Few intervention trials have examined the effect of individual marine n-3 PUFA, as most intervention trials have examined a combination of EPA and DHA since these are found together in seafood. However, one large intervention trial in a Japanese population used a supplement of purified EPA and found a significant reduction in major cardiovascular events, suggesting cardioprotective effects of EPA independently of other marine n-3 PUFA (126). Thus, it has been suggested that individual marine n-3 PUFA have both shared and important differential effects (133).

Several mechanisms have been suggested to explain the cardio-protective properties attributed to marine n-3 PUFA in general. N-3 PUFA are incorporated into cell membrane phospholipids, where they may affect membrane function, cell signalling, arrhythmic properties, and a variety of other processes (14,65,134). These mechanisms may explain a possible antithrombotic (126,135,136) and/or antiarrhythmic (44,60,61,137) effect of marine n-3 PUFA that may reduce risk of CHD. In addition, anti-inflammatory properties have been reported (11,134,138), possibly inferring a stabilising effect of marine n-3 PUFA on human atherosclerotic plaques (62,67) that may decrease the risk of plaque rupture and thus CHD events. Finally, marine n-3 PUFA also lower plasma triglycerides (139), increase serum adiponectine (140,141), impair platelet and leukocyte reactivity, lower heart rate, and may slightly reduce blood pressure (11,14,63,65).

One of the key mechanisms linking the 5-LOX pathway to the development of atherosclerosis may be attributed to the formation of leukotrienes and their pro-inflammatory properties. Leukotrienes increase vascular permeability and act as potent chemo-attractants among other pro-inflammatory functions (69,70,74). Furthermore, a high expression of multiple 5-LOX enzymes has been found in human

atherosclerotic plaques and levels of the same enzymes have been linked to the stage of plaque development (82,83). Genetic association studies have also provided evidence of a link between the 5-LOX pathway and atherosclerotic disease as discussed earlier. Especially, the *ALOX-5* tandem repeat polymorphism has demonstrated a functional relationship between genetic variants leading to altered enzymatic activity and ultimately affecting the risk of MI. In this regard, Study III adds to the body of evidence in support of this hypothesis.

The functional relationship between n-3 PUFA and the 5-LOX pathway is evident as EPA is a substrate for the 5-LOX enzymes competing with AA (11,14). Importantly, EPA generally leads to the formation of far less pro-inflammatory leukotrienes compared with leukotrienes derived from AA (72,73). In accordance with this, it has been proposed that anti-inflammatory properties of n-3 PUFA intake may be explained by the incorporation of n-3 PUFA, mainly EPA, at the expense of AA in cell membrane phospholipids, thereby causing a shift in the leukotriene production towards less pro-inflammatory leukotrienes among other effects (142). In Study IV, our results indicated an interaction (although not statistically significant) between fatty acid substrates for the 5-LOX enzymes and the ALOX-5 tandem repeat polymorphism (rs59439148), which is in line with the hypothesis that the differential roles of marine n-3 PUFA and AA in atherosclerotic disease states may, at least partly, be explained by the formation of less pro-inflammatory leukotrienes derived from EPA compared with AA. In this respect, the study provides the characteristics of a Mendelian randomisation study adding mechanistic evidence for a role of EPA and AA in MI through the 5-LOX system.

General comments on the study design and generalisability

Specific limitations related to the individual studies are mentioned in "Chapter 5. Studies" and in the papers included in the appendix. Here, a few general thoughts on the prospective design and generalisability will be provided.

First of all, we used an observational design. The Diet, Cancer and Health study is a prospective cohort study which has been carefully designed and well described in hundreds of studies. Compared to case-control studies, the prospective cohort design holds some advantages. For instance, the participants were unaware of future disease when entering the cohort, which minimises the possibility of information bias or that the knowledge of disease has affected the lifestyle and habits of the participants before baseline information was collected. Furthermore, selection bias is minimal in cohort studies and usually caused by differentiated loss of participants during follow-up between the case group and cohort as a whole. In this regard, the cohort has been well accounted for, and loss to follow-up was minimal. Thus, selection bias was unlikely to be substantial in the studies.

It is important to consider the generalisability of the studies. The Diet, Cancer and Health study included middle-aged subjects (50-64 years at baseline) without previous

history of cancer, and for the present studies we excluded subjects with a previous history of MI as well. Of all the subjects that were invited, 35% agreed to participate, so it is possible that the study cohort is somewhat different from the general population that was invited. Notably, it was found that the participants in the study had a better socio-economic status than the invited group all together (110). This is, however, a common problem in cohort studies, and it is possible that the group that did not participate could have contributed a relatively high or perhaps more likely a relatively low intake of fish, which would then have resulted in a wider range of the exposure in Study I, II & IV and, in turn, a more pronounced effect of the exposure. It is therefore possible that the non-acceptance group, given that they provide more extreme levels of the exposure, would augment the associations between the exposure and outcome. Given the lower socio-economic status, it is also plausible that the non-acceptance group would have a higher morbidity and prevalence of various risk factors for MI. However, it is less likely that this would influence the associations between fish intake and MI substantially.

CHAPTER 7. CONCLUSIONS AND PERSPECTIVES

Overall conclusions

We examined the association of fish consumption and adipose tissue content of marine n-3 PUFA with incident MI, and in general, the results showed a modest inverse association between incident MI and consumption of fatty fish as well as the content of EPA in adipose tissue. In contrast, no effect was demonstrated from consumption of lean fish. Thus this thesis provide further support for the hypothesis that consumption of fatty fish and n-3 PUFA is beneficial in primary prevention of MI.

Next, we examined polymorphisms in the 5-LOX pathway and found some individual SNPs to be associated with MI. Furthermore, haplotypes identified in earlier studies were not associated with MI in our population. Taken together, our results suggest that the 5-LOX pathway may play a role in the risk of MI. However, when comparing our findings to those of other studies examining polymorphisms in the 5-LOX pathway, no evident patterns was found, and studies on the pathway have failed to identify strong genetic determinants and functional polymorphisms in most of the candidate genes.

We then explored potential interaction between substrates for the 5-LOX pathway and variants of a tandem repeat polymorphism in the *ALOX-5* gene. The results suggested a functional relationship between substrate availability and the genotype, collectively affecting the risk of MI. However, though our results indicated interaction, the study was inconclusive because of lack of power to detect interaction at the level of statistical significance. Thus, we cannot completely rule out that our results in Study IV were coincidental, but nevertheless, the study illustrates the importance of complex environment-gene interactions and that these should be carefully considered.

Perspectives

CVD remains a leading cause of death worldwide, despite markedly improved treatment of acute coronary syndromes and prevention of CVD, during the last two decades. Thus, improvement of both primary and secondary prevention of CVD is of major importance. In this regard, marine n-3 PUFA have long been recommended for the secondary prevention of CHD. Evidence from cohort studies have generally supported the opinion that marine n-3 PUFA may have a role in primary prevention of CHD as well, which was supported by the results from this thesis. However, in our cohort, the associations were modest compared to early prospective cohort studies. Furthermore, recent secondary prevention trials have reported neutral associations between fish intake and clinical endpoints, questioning the beneficial effects of n-3 PUFA, and a gap between the evidence from primary and secondary prevention trials

have become more evident. All together, these discrepancies have stimulated a renewed interest and discussion on dietary recommendations with regards to marine n-3 PUFA, and future studies should aim at clarifying these discrepancies between primary and secondary prevention trials. For instance, it would be of interest to look into aspects of dietary substitution to help explain differences between dietary fish intake and fish oil supplements. Also, the role of n-3 PUFA in the context of aggressive medical therapy, which might influence on the mechanisms of action for n-3 PUFA, needs further investigation. Finally, differential effects for individual n-3 PUFA have been suggested, but trial evidence is limited especially for DPA.

The 5-LOX pathway is an inflammation related pathway that has been implicated in atherothrombotic disease, and genetic association studies have provided some support for this relationship. However, important discrepancies exist between studies and although some of these discrepancies may be explained by differences in the study design and the populations examined, strong genetic determinants remains to be confirmed. Importantly, some studies have found evidence of diet-gene interaction by substrates for the 5-LOX enzymes, which should be carefully considered in future studies examining the pathway. Another aspect that may be important, is the possibility that several common polymorphisms may interact downstream the enzymatic pathway, to influence the production of leukotrienes all together. This have lead some authors to suggested that a "pathway approach" may be more appropriate, and a study examining aspects of gene-gene interaction in our data, is currently being planned. Despite inconsistencies in genetic association studies, the 5-LOX pathway remains an essential inflammatory pathway implicated in several inflammatory diseases such as atherosclerosis and bronchial asthma, and future research may provide new targets for treatment of theses widespread diseases, via the 5-LOX pathway or leukotrienes.

CHAPTER 8. ENGLISH SUMMARY

Cardiovascular disease (CVD) remains a leading cause of death worldwide and atherosclerosis has been recognised as an essential component of the disease. The mechanisms leading to atherosclerosis has not been fully uncovered, but several researchers has suggested that inflammatory processes in the vessel wall plays an important role. In this setting, researchers have developed an interest in the leukotrienes that have been associated with atherothrombotic disease. The leukotrienes are highly pro-inflammatory substances formed by specific n-3 and n-6 fatty acids, but the leukotrienes formed by n-3 fatty acids are considerably less pro-inflammatory compared to leukotrienes formed from n-6 fatty acids. The samme fatty acids has been implicated in CVD, where n-3 fatty acids from fatty fish has been associated with a lower risk of CVD, whereas other studies have suggested that n-6 fatty acids may be associated with higher risk of CVD. Thus, it is plausible that the dietary intake of specific n-3 and -6 fatty acids may influence the risk of atherosclerotic disease through their formation of different types of leukotrienes.

In his PhD-study we investigated the association between marine n-3 fatty acids, evaluated from dietary intake of fish and adipose tissue content of marine n-3 fatty acids, and myocardial infarction (MI) in two independent studies. Secondly, we investigated genetic variants in four genes involved in the formation of leukotrienes, and how the genotypes were associated with MI. Finally, we investigated the interplay between a specific genetic variant and adipose tissue content of n-3 and n-6 fatty acids in relation to MI. The studies were based on a large Danish cohort study, the Diet, Cancer and Health Study, that included more than 57.000 participants and currently has been followed for more than 17 years. During this period we identified and validated 3.089 cases with incident MI.

In the two studies examining marine n-3 fatty acids, we found a moderate lower risk of MI in the groups with a high intake or content of n-3 fatty acids, which is in line with the current opinion among many researchers, that consumption of fatty fish and n-3 fatty acids is beneficial in the prevention of CVD. Furthermore, our study investigating genetic variants in the leukotriene pathway found some variants to be associated with MI, and collectively, the study suggested that the leukotriene pathway may play a role in the risk of MI. Finally, examining the interaction between a specific polymorphism in the leukotriene pathway and adipose tissue content of n-3 and -6 fatty acids, the study indicated that the effect of the polymorphism was modified by the content of the respective fatty acids. Thus, a high content of the n-3 fatty acid attenuated the effect of the variant genotype, while a high content of the n-6 fatty acid augmented the effect of the variant.

CHAPTER 9. DANSK RESUMÉ

Hjerte-kar-sygdom er fortsat en af de hyppigste dødsårsager på verdensplan og åreforkalkning er en central del af sygdomsprocessen. Mekanismerne bag udviklingen af åreforkalkning er endnu ikke fuldt klarlagt, men flere forskere har foreslået, at inflammatoriske processer i karvæggen spiller en afgørende rolle. I denne forbindelse findes en gruppe af stoffer med stærke inflammationsfremmende egenskaber, kaldet leukotriener, som er blevet kædet sammen med risiko for hjerte-kar-sygdom. Disse dannes ud fra bestemte n-3 og -6 fedtsyrer, men leukotrienerne, der dannes ud fra en n-3 fedtsyre, er markant mindre pro-inflammatoriske end dem, der dannes ud fra en n-6 fedtsyre. De samme fedtsyrer er også blevet kædet sammen med hjerte-kar-sygdom, hvor n-3 fedtsyrer, der findes i fede fisk, er blevet fundet at have en beskyttende virkning på hjerte-kar-sygdom. Derimod har andre undersøgelser vist, at n-6 fedtsyrer kan øge risikoen for hjerte-kar-sygdom. Det er således muligt, at indtaget af n-3 og -6 fedtsyrer gennem kosten, kan have betydning for udviklingen af åreforkalkning gennem dannelsen af forskellige typer af leukotriener.

I dette ph.d.-studie undersøgte vi i to studier, hvorledes indtaget af fisk og indholdet af n-3 fedtsyrer i fedtvæv var associeret med akut myokardieinfarkt. Dernæst undersøgte vi i et tredje studie variationer i generne, der er involveret i dannelsen af leukotrienerne, og hvordan genotypen var associeret med akut myokardieinfarkt. Til slut undersøgte vi samspillet mellem en udvalgt gen-variant og indholdet af n-3 og -6 fedtsyrer i fedtvæv i relation til akut myokardieinfarkt. Studierne tog udgangspunkt i en stor dansk befolkningsundersøgelse, Kost, kræft og helbred som inkluderede mere end 57.000 personer, og som på nuværende tidspunkt er blevet fulgt i mere end 17 år i gennemsnit. I denne periode identificerede og validerede vi 3.089 personer med akut myokardieinfarkt.

De to første studier fandt en moderat lavere risiko for akut myokardieinfarkt i grupperne med højt indtag af fed fisk eller højt indhold af n-3 fedtsyrer i fedtvæv, hvilket støtter den generelle opfattelse at indtaget af n-3 fedtsyrer fra fisk har en beskyttende virkning mod hjerte-kar-sygdom. Dernæst viste vores undersøgelse af genetiske varianter i leukotriensystemet, at nogle af polymorfierne var associeret med risikoen for akut myokardieinfarkt og samlet set viste studiet, at leukotriensystemet muligvis har en betydning for risikoen for akut myokardieinfarkt. I studie IV, hvor vi undersøgte vekselvirkning mellem en genetisk polymorfi i leukotriensystemet og n-3 og -6 fedtsyrer, indikerede resultaterne at effekten af polymorfien blev modificeret af n-3 og -6 fedtsyrerne, således at et højt indhold af n-3 fedtsyren i fedtvæv svækkede effekten af varianterne hvorimod et højt indhold af n-6 fedtsyren i fedtvæv øgede effekten af varianterne.

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APPENDICES

Appendix A. Paper I.....1

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Appendix A. Paper I

British Journal of Nutrition (2016), 116, 167–177 doi:10.1017/S000711451600180X

Association of fish consumption and dietary intake of marine *n*-3 PUFA with myocardial infarction in a prospective Danish cohort study

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(Submitted 23 January 2016 – Final revision received 31 March 2016 – Accepted 5 April 2016)

Abstract

Several studies have investigated the potential benefits of marine n-3 PUFA in CVD, generally suggesting a lower risk of CHD. However, recent trials have questioned these results. This study investigated the association of fish consumption with dietary intake of marine n-3 PUFA with incident myocardial infarction (MI). In a Danish cohort study, 57 053 subjects between 50 and 64 years of age were enrolled from 1993 to 1997. From national registries, we identified all cases of incident MI. Dietary fish consumption was assessed using a semi-quantitative food questionnaire, including twenty-six questions regarding fish intake. In addition, we calculated the intake of total and individual marine n-3 PUFA. During a median follow-up of 17·0 years, we identified 3089 cases of incident MI. For both men and women, a high intake of fatty fish was inversely related to incident MI. Thus, when comparing the highest and the lowest quintile of fatty fish intake, we found a 12 % lower relative risk of MI in men (hazard ratio (HR) 0·88; 95 % CI 0·77, 1·00) and a 22 % lower relative risk in women (HR 0·78; 95 % CI 0·63, 0·96) after adjustments. For women, similar associations were observed for individual and total marine n-3 PUFA. In contrast, intake of lean fish was not associated with MI. In conclusion, incident MI was inversely related to a high intake of fatty fish, but not lean fish. However, test for trends across quintiles was not statistically significant. In general, this study supports the view that consumption of fatty fish may protect against MI.

Key words: Myocardial infarction: Cohort studies: Marine n-3 PUFA: Fish consumption

The potential benefit of marine n-3 PUFA on CHD has been extensively examined in both observational studies and clinical trials, with the majority of studies favouring a beneficial effect of n-3 PUFA[1–4].

Thus, several prospective cohort studies have reported an inverse relationship between fish consumption and CHD[1,5–14]. However, other studies have not supported these findings[15,16]. Most epidemiological studies have evaluated the dietary intake of fish using questionnaires, but relatively few of these studies have differentiated between different types of fish and their content of marine n-3 PUFA. In this study, we evaluated fish intake using detailed and validated FFQ, allowing us to differentiate between consumption of fatty and lean fish, and additionally to calculate the intake of total and individual marine n-3 PUFA.

Large secondary prevention trials have been performed evaluating dietary interventions with fish servings or supplementation with fish oil capsules. Although earlier trials such as the diet and reinfarction trial[17], Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI) Prevenzione[18] and Japan EPA Lipid Intervention Study trials[19] have demonstrated a reduction in CHD events following consumption of marine n-3 PUFA, more recent trials have not supported these findings[20–22]. Controversy therefore still remains regarding the potential benefits from fish consumption and n-3 PUFA supplements in the prevention of CHD, and guidelines are currently under review.

In this large Danish cohort study, including 3089 validated cases of incident myocardial infarction (MI), we investigated the association of fish consumption and dietary intake of marine n-3 PUFA with incident MI. The present study is one of the largest cohort studies on fish consumption and MI, and notably the study includes a relatively high number of female cases.

Methods

Study population and design

The Danish Diet, Cancer and Health study is a prospective cohort study, which has been described in detail previously[23].

Abbreviations: DPA, docosapentaenoic acid; ICD; International Classification of Disease; MI; myocardial infarction.

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In brief, 160 725 persons aged 50–64 years were invited between December 1993 and May 1997. Inclusion criteria were as follows: born in Denmark, living in the urban areas of Copenhagen and Aarhus and not registered with a cancer diagnosis in the Danish Cancer Registry at the time of invitation. A total of 57 053 persons accepted the invitation and were enrolled into the study. Participants registered with a previous MI or cardiac arrest were excluded. If a cancer diagnosis was reported, which was not already recorded in the Cancer Registry at the time of invitation, participants were excluded in line with the intention-to-include criteria. At baseline, each participant filled in a detailed questionnaire on diet, lifestyle, socio-economic status and medical history. Data from dietary questionnaires were analysed in a traditional cohort design including the entire cohort. Participants were followed-up until July 2013. The present study was conducted according to the Helsinki Declaration and approved by the regional Ethics Committees.

Dietary assessment

At baseline, participants filled in a detailed 192-point, semi-quantitative FFQ, including twenty-six specific questions regarding the intake of fish and food products containing fish. The questionnaire has been validated and described in detail previously[24]. In short, participants were asked to estimate their daily intake of foods in natural units such as pieces of fruits, slices of bread and glasses of different drinks. For mixed dishes and meals, sex-specific portion sizes were calculated using data from a calibration study. By multiplying the frequencies of intake by the portion size, the individual average intake in g/d of all foods and nutrients was calculated. Different species of fish were categorised as either lean or fatty depending on their content of *n*-3 PUFA, below or above 1 g/100 g, respectively. Fatty fish mainly comprised herring, salmon, trout and mackerel, whereas lean fish comprised mainly plaice, flounder and cod[7].

The dietary intakes of specific nutrients including total and individual marine *n*-3 PUFA – EPA, docosapentaenoic acid (DPA) and DHA – were calculated using FoodCalc software (www.ibt.ku.dk/jesper/foodcalc) based on Danish Food Composition Tables.

Outcome assessment

We identified all participants in the cohort who were registered with an incident diagnosis of MI in the Danish National Patient Registry and/or the Danish Causes of Death Registry, according to the International Classification of Disease (ICD) 8 (410.00–410.99) or ICD-10 (I21.0–I21.9) coding, during the study period. Furthermore, all cases of cardiac arrest (ICD-8: 427.27 or ICD-10: I46.0–I46.9) were included if the arrest was considered to be of cardiac origin after validation in each individual case. The Danish National Patient Registry[25] contains information on diagnoses and interventional procedures in relation to all hospital ward admissions since 1977, whereas all visits to outpatient clinics and emergency rooms have been registered since 1995. The Danish Causes of Death Registry contains diagnoses for all deaths since 1943. Patients were registered in both registries in accordance with ICD-8 until 1 January 1995 and subsequently according to ICD-10. An earlier study from our Department validated the MI diagnosis from baseline through 2003 by complete review of all medical records, and found a positive predictive value >92 % when the diagnoses were obtained from a hospital ward[26]. All validated cases of MI from the validation study were readily accepted as cases for the present study. From January 2004 through July 2013, all participants with an incident MI diagnosed from a ward were accepted as cases without further validation. All other diagnoses of incident MI and cardiac arrest were validated by reviewing a complete list of diagnoses and interventional procedures recorded in the Danish National Patient Registry for each potential case. Cases of incident MI were categorised as fatal or non-fatal depending on their vital status 28 d after the event. Vital status was obtained using the Civil Registration System.

Statistics

Exposure variables were categorised into quintiles based on the cohort distribution. A test for trend across quintiles was carried out. Furthermore, we also evaluated the exposure variables as continuous variables using restricted cubic splines with three knots.

Measures of association were assessed using Cox proportional hazards multivariate regression models with age as the time axis and delayed entry. Analyses were conducted separately for men and women. Participants were treated as at risk from baseline until either MI, death, emigration or end of follow-up occurred.

To address potential confounding, we adjusted for traditional risk factors of MI (model A2), including smoking habits (never, former or current (<15, 15–25, >25 g/d) smoker), BMI (kg/m^2), waist circumference (cm), physical activity (h/week), alcohol intake (g/d), educational level (basic school, higher education: 1–3 or >3 years) and, for women, menopausal status (pre or postmenopausal). Second, we adjusted for either medical history (model B) or additional dietary covariates (model C) in separate models. Regarding medical history, we adjusted for history of diabetes mellitus (yes/no), hypertension (yes/no) or hypercholesterolaemia (yes/no), including participants on antihypertensive medications or lipid-lowering drug treatment, respectively. Adjustment for additional dietary variables depended on the exposure variable (details specified in the relevant tables). All continuous variables were included in the models as restricted cubic splines with five knots. Potential confounders were selected a priori based on current knowledge of risk factors for MI.

The proportional hazards assumption was checked by visual inspection of log–log plots and by evaluation of scaled Schoenfeld residuals, with no significant violations. Estimates with P values (two-tailed) <0·05 were considered statistically significant. STATA, version 13.1 (StataCorp. LP) was used for statistical analysis.

Results

The Diet, Cancer and Health cohort included a total of 57 053 participants. From these, an initial 1506 were excluded when

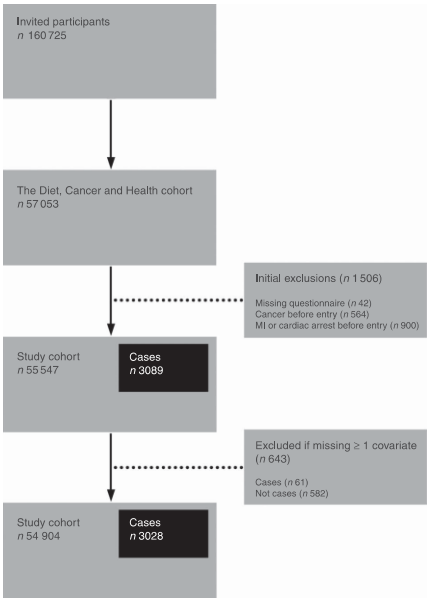

Fig. 1. Flow chart of cohort selection process. MI, myocardial infarction.

baseline questionnaires were missing (n 42) or when participants were identified with a cancer diagnosis in the Danish National Patient Registry before baseline (n 564). We also excluded participants with previous MI or cardiac arrest before inclusion (n 900). In our study population, we identified 3089 cases of incident MI during a median follow-up time of 17·0 years. After case verification, we excluded subjects for whom information on one or more covariates used in the adjusted analyses was missing (n 643). Thus, 3028 cases were included in the final analyses (Fig. 1).

Baseline risk factors for CHD were generally more prevalent in cohort members who experienced a MI during follow-up ('cases') than in the cohort as a whole (Table 1). Accordingly, we observed a larger proportion of men, higher age, BMI and waist circumference, and a larger proportion of smokers among cases. In contrast, cases had lower weekly physical activity and lower educational level compared with the cohort. With regard to medical history, more cases suffered from hypertension, hypercholesterolaemia and diabetes mellitus. Consumption of fruits and vegetables was lower among cases, whereas we did not find any marked differences between cases and the cohort with regard to fish consumption and use of fish oil supplements. Baseline characteristics were also evaluated by the intake of fatty and lean fish for men and women separately, in order to describe potential confounders. Data are included in the online Supplementary Material (online Supplementary Table S1·2).

Fish consumption and incident myocardial infarction

The median intake of fatty fish was 15·1 and 12·2 g/d for men and women, respectively, whereas the median intake of lean fish was 24·4 and 21·0 g/d for men and women, respectively, with minor variations between cases and cohort (Table 1).

In Table 2, we present hazard ratios (HR) for the association between fish consumption and incident MI distributed by quintiles. For men, fatty fish intake was inversely associated with MI in the four higher quintiles compared with the lowest. The association was attenuated by adjustment for traditional risk factors for MI, but the trend remained for the highest quintile compared with the lowest when adjusting for traditional risk factors and medical history (HR 0·88; 95 % CI 0·77, 1·00). For women, we found an inverse association between the highest quintile of fatty fish consumption and MI, which remained statistically significant after adjustment for traditional risk factors and medical history (HR 0·78; 95 % CI 0·63, 0·96). Adjustment for dietary variables did not affect the measures of association substantially; however, results for model C were not statistically significant. The test for trends across quintiles was not statistically significant in the adjusted models, and a linear dose–response relationship could not be demonstrated. No consistent associations were seen between consumption of lean fish and MI, for neither men nor women.

In addition, fish intakes were evaluated as continuous variables, modulated as restricted cubic splines with three knots, suggesting an inverse trend between intake of fatty fish and MI for both men and women, whereas lean fish was not associated with MI (Fig. 2).

Dietary intake of marine n-3 PUFA and incident myocardial infarction

We also examined the intakes of individual and total marine n-3 PUFA (EPA, DPA and DHA). The median intake of total marine n-3 PUFA was 0·7 and 0·6 g/d for men and women, respectively. As expected, intake of fatty fish was highly correlated with the calculated intake of marine n-3 PUFA (r 0·93, $P < 0·001$). For men, we observed a trend towards a negative association between EPA, DHA and total marine n-3 PUFA when comparing the highest and lowest quintiles; however, the associations were modest and not statistically significant for any of the models (Table 3). Results were generally more consistent, towards an inverse association, between marine n-3 PUFA and incident MI for women. Accordingly, intake of total and individual marine n-3 PUFA was inversely associated with MI. However, only model B, adjusting for traditional risk factors and medical history, remained statistically significant when comparing the fifth quintile with the first (HR 0·81; 95 % CI 0·66, 0·99). As with intake of fatty fish, the test for trends was not statistically significant across quintiles in the adjusted analyses.

The associations between total marine n-3 PUFA and MI, modulated by restricted cubic splines, are presented in Fig. 2. For women, we found a trend towards an inverse association with MI, whereas for men the association was less strong.

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Table 1. Baseline characteristics of the cohort and cases with myocardial infarction*
(Medians and 10th and 90th percentiles (continuous variables) and numbers and percentages (categorical variables))

	Men				Women			
	Cohort (*n* 25 913)		Cases (*n* 2136)		Cohort (*n* 28 991)		Cases (*n* 892)	
Variables	Medians	10th; 90th percentile	Medians	10th; 90th percentile	Medians	10th; 90th percentile	Medians	10th; 90th percentile
Age (years)	55·9	51·2; 63·3	57·7	51·7; 63·9	56·2	51·2; 63·2	59·3	52·4; 64·2
Physical activity (h/week)	2·0	0·0; 8·0	2·0	0·0; 8·0	2·5	0·0; 8·0	2·0	0·0; 7·0
BMI (kg/m^2)	26·1	22·5; 31·1	26·9	23·2; 32·2	24·8	20·8; 31·2	25·9	20·9; 33·3
Waist circumference (cm)	95·0	84·0; 108·5	97·0	86·0; 112·0	80·0	69·0; 97·0	84·0	70·0; 102·0
Smoking								
Never smoker								
n		6772		389		12 719		242
%		26·1		18·2		43·9		27·1
Former smoker								
n		8892		631		6797		173
%		34·3		29·5		23·5		19·4
<15 g/d								
n		2744		265		4421		198
%		10·6		12·4		15·3		22·2
15–25 g/d								
n		4522		509		4305		233
%		17·5		23·8		14·9		26·1
>25 g/d								
n		2983		342		748		46
%		11·5		16·0		2·6		5·2
Educational level								
Basic school								
n		8867		923		9043		392
%		34·2		43·2		31·2		44·0
Higher education, 1–3 years								
n		10 814		796		14 556		413
%		41·7		37·3		50·2		46·3
Higher education, >3 years								
n		6232		417		5392		87
%		24·1		19·5		18·6		9·8
Menopausal status								
Postmenopausal								
n		–		–		16 975		617
%		–		–		58·6		69·2
Premenopausal								
n		–		–		9022		158
%		–		–		31·1		17·7
Medical history								
Hypertension								
n		3762		472		4984		277
%		14·5		22·1		17·2		31·1
Hypercholesterolaemia								
n		2005		253		1762		13·1
%		7·7		11·8		6·1		117
Diabetes mellitus								
n		677		115		435		39
%		2·6		5·4		1·5		4·4
Dietary variables (g/d)								
Fruits (excluding juice)	117·7	27·0; 324·7	111·0	25·1; 313·2	172·4	49·1; 417·4	160·6	37·5; 399·2
Vegetables (including juice, excluding potatoes)	151·8	62·7; 291·4	138·8	56·7; 277·5	171·9	69·8; 329·4	149·2	54·5; 318·3
Alcohol	19·4	3·6; 62·6	18·2	2·5; 62·7	9·3	1·0; 34·5	6·5	0·5; 31·9
Total energy intake (MJ/d)	9·9	7·1; 13·6	9·8	7·1; 13·4	8·0	5·7; 11·2	7·9	5·5; 11·1
Dietary fish intake								
Fatty fish	15·1	4·4; 37·8	14·9	4·1; 37·2	12·2	3·6; 31·8	11·6	2·6; 31·0
Lean fish	24·4	10·1; 50·0	24·2	10·4; 51·2	21·0	9·2; 42·6	20·6	8·5; 43·3
Total fish	41·7	17·7; 81·5	41·1	17·3; 82·3	35·2	15·1; 69·8	34·4	12·8; 67·9
EPA (mg/d)	177·3	68·7; 379·1	176·5	65·7; 376·6	147·5	56·1; 321·7	145·2	48·3; 317·7
DPA (mg/d)	82·0	47·3; 134·5	82·7	46·5; 136·5	61·8	33·7; 105·1	62·3	31·6; 103·4
DHA (mg/d)	430·7	189·2; 866·1	428·8	179·3; 855·4	358·3	151·7; 726·9	354·5	132·8; 705·5
Total marine *n*-3 PUFA (mg/d)	692·2	313·7; 1370·1	686·6	295·2; 1355·2	569·2	248·8; 1148·3	563·8	214·8; 1115·8
Fish oil supplements								
n		4116		295		5073		295
%		15·9		16·4		17·5		18·8

DPA, docosapentaenoic acid.
* Comparison of baseline characteristics for the cohort as a whole (including both cases and non-cases) and cases with myocardial infarction separately.

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Table 2. Association of dietary fish intake and myocardial infarction according to type of fish* (Hazard ratios (HR) and 95 % confidence intervals)

	Men										Women									
	Model A1†		Model A2‡		Model B§		Model CII				Model A1†		Model A2‡		Model B§		Model CII			
	HR	95 % CI	HR	95 % CI	HR	95 % CI	HR	95 % CI			HR	95 % CI	HR	95 % CI	HR	95 % CI	HR	95 % CI		
Intake of fatty fish									Intake of fatty fish											
Q1 (0–8 g)	1		1		1		1		Q1 (0–6 g)		1		1		1		1			
Q2 (>8–13 g)	0·88	0·77, 1·00	0·89	0·79, 1·03	0·90	0·79, 1·03	0·91	0·80, 1·04	Q2 (>6–10 g)		0·86	0·70, 1·06	0·95	0·77, 1·16	0·95	0·77, 1·16	0·96	0·78, 1·18		
Q3 (>13–18 g)	0·84	0·73, 0·96	0·89	0·78, 1·01	0·89	0·78, 1·02	0·90	0·79, 1·03	Q3 (>10–15 g)		0·85	0·69, 1·04	0·96	0·78, 1·17	0·93	0·76, 1·15	0·98	0·80, 1·21		
Q4 (>18–28 g)	0·82	0·72, 0·94	0·90	0·79, 1·03	0·90	0·78, 1·03	0·92	0·80, 1·06	Q4 (>15–23 g)		0·90	0·74, 1·10	1·07	0·87, 1·31	1·04	0·85, 1·27	1·11	0·90, 1·36		
Q5 (>28 g)	0·83	0·72, 0·94	0·91	0·79, 1·04	0·88	0·77, 1·00	0·93	0·81, 1·07	Q5 (>23 g)		0·69	0·56, 0·85	0·83	0·67, 1·03	0·78	0·63, 0·96	0·86	0·69, 1·08		
P_{trend}		P<0·01		P=0·18		P=0·08		P=0·38	P_{trend}			P<0·01		P=0·32		P=0·10		P=0·57		
Intake of lean fish									Intake of lean fish											
Q1 (0–14 g)	1		1		1		1		Q1 (0–13 g)		1		1		1		1			
Q2 (>14–21 g)	1·07	0·94, 1·23	1·12	0·98, 1·29	1·13	0·99, 1·29	1·14	1·00, 1·31	Q2 (>13–18 g)		0·94	0·77, 1·16	1·00	0·82, 1·23	1·01	0·82, 1·24	1·03	0·84, 1·27		
Q3 (>21–28 g)	1·05	0·92, 1·21	1·11	0·97, 1·27	1·10	0·96, 1·26	1·14	0·99, 1·30	Q3 (>18–24 g)		0·87	0·71, 1·07	0·95	0·77, 1·17	0·93	0·76, 1·15	0·98	0·79, 1·21		
Q4 (>28–39 g)	1·01	0·89, 1·16	1·09	0·95, 1·25	1·07	0·94, 1·23	1·12	0·97, 1·29	Q4 (>24–33 g)		0·90	0·73, 1·10	0·99	0·81, 1·22	0·98	0·80, 1·21	1·04	0·84, 1·29		
Q5 (>39 g)	1·04	0·91, 1·19	1·07	0·93, 1·23	1·05	0·91, 1·20	1·12	0·97, 1·29	Q5 (>33 g)		0·89	0·72, 1·09	0·95	0·77, 1·16	0·93	0·75, 1·14	0·99	0·79, 1·24		
P_{trend}		P=0·91		P=0·51		P=0·78		P=0·21	P_{trend}			P=0·23		P=0·61		P=0·44		P=0·98		

Q, quintiles.
* *P* values test the hypothesis of no trend across quintiles.
† Model A1: crude analysis.
‡ Model A2: adjusted for traditional risk factors including smoking, BMI, waist circumference, physical activity, alcohol intake, educational level and menopausal status (women).
§ Model B: adjusted as model A2 with additional covariates: history of diabetes mellitus, hypertension and hypercholesterolaemia.
‖ Model C: adjusted as model A2 with additional dietary covariates: total energy intake, intake of fruits and vegetables and intake of nuts.

Fatal myocardial infarction

As a secondary analysis, we evaluated the association of fish consumption and intake of marine *n*-3 PUFA with fatal MI (Table 4). After validation, 580 cases (424 men and 156 women) were categorised as having a fatal MI. Generally, the same tendency towards a negative association for fatty fish intake was observed; however, the CI were broader, and the associations were not statistically significant.

Discussion

In this Danish prospective cohort study conducted in middle-aged men and women, we examined fish consumption and dietary intake of marine *n*-3 PUFA in relation to risk of MI. Dietary intake of fatty fish was inversely related to incident MI among men and women when comparing the highest and lowest quintiles, although the association only remained statistically significant for women after multivariate adjustments. For women, the same association was shown for total and individual marine *n*-3 PUFA with no material differences between EPA, DPA and DHA.

Moreover, two studies have been published previously, based on the Diet Cancer and Health cohort, examining the association of fish intake with adipose tissue marine *n*-3 PUFA content in participants with Acute Coronary Syndrome (ACS)[6,7]. In the present study, the follow-up period was extended and the number of cases was larger providing considerably more strength to explore the associations in both men and women. Thus, Bjerregaard *et al.*[6] found an inverse relationship between intake of fatty fish and ACS in men, suggesting a 32 % lower risk from eating a moderate amount of fatty fish, whereas Joensen *et al.*[7] demonstrated a lower and dose-dependent risk of ACS in men with a high content of marine *n*-3 PUFA in adipose tissue. Findings in women were inconsistent and non-significant in both studies[6,7]. In the present, substantially larger, study, we also demonstrated an inverse relationship in men, but the associations were generally attenuated during the longer follow-up period, and our results concerning fatty fish intake suggested a more modest relative risk of 0·88 when comparing the lowest and the highest quintiles in men. In contrast, our findings in women showed a consistent pattern compared with the earlier studies and suggested a 22 % lower risk from high fish intake comparing the highest quintile with the lowest.

There is generally strong supportive evidence from epidemiological studies[5–12,15] and secondary intervention trials[17–19] that intake of fish and marine *n*-3 PUFA is associated with a lower risk of CHD and sudden cardiac death. Thus, in 2004, He *et al.* conducted a meta-analysis of prospective cohort studies, suggesting that one fish serving/week is associated with a 15 % lower risk of fatal CHD and 2–4 servings/week with a 23 % lower risk. Today, both the American Heart Association[27] and the European Society of Cardiology[28] recommend a daily intake of marine *n*-3 PUFA of 1 g/d for secondary prevention of CHD.

Recent clinical trials have questioned the beneficial effects of *n*-3 PUFA supplements[20–22], and several reasons have been

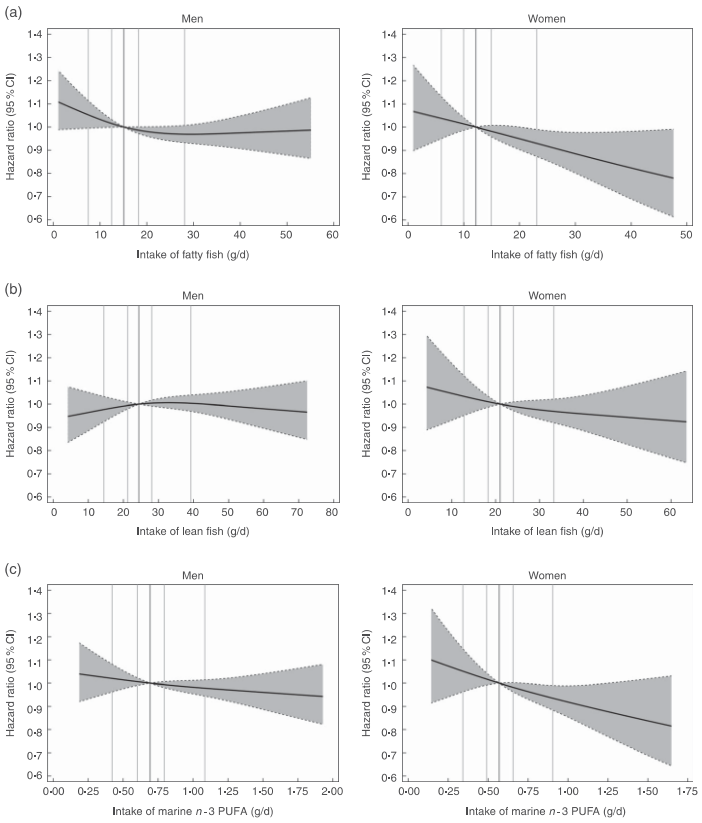

Fig. 2. Spline curves showing hazard ratios and 95 % CI (▨) for the associations between (a) fatty fish, (b) lean fish, (c) total marine *n*-3 PUFA and incident myocardial infarction. Data presented for model B, adjusting for traditional risk factors and medical history, with the median dietary intake as reference. ——, Intake by quintiles.

proposed to explain these findings. First of all, methodological variations, different populations and different interventions might explain some of the differences. Notably, the recent trials were all conducted in populations with aggressive background therapy with antihypertensive, antiplatelet and lipid-lowering drugs, thereby diminishing the cardio-protective potential of *n*-3 PUFA. Furthermore, the effect of *n*-3 PUFA was mainly seen for fatal CHD, whereas most recent trials have focused on non-fatal or composite end points for CHD, and finally the background dietary fish intake was generally higher in recent trials in accordance with rising public awareness of a healthy lifestyle[29]. In line with this, a recent meta-analysis reported a modest effect of marine *n*-3 PUFA on cardiovascular end points[3].

Several mechanisms have been suggested to explain the cardio-protective properties attributed to marine *n*-3 PUFA[2,4,30]. These mechanisms may explain possible antithrombotic[19,31,32] and/or antiarrhythmic[18,33,34] effects of marine *n*-3 PUFA that may reduce the risk of CHD. In addition, anti-inflammatory properties have been reported, possibly inferring a stabilising effect of marine *n*-3 PUFA on human atherosclerotic plaques[35,36] that may decrease the risk of plaque rupture, and thus MI and CHD. Finally, marine *n*-3 PUFA also lower plasma TAG[37], increase serum adiponectin[38,39] and may slightly reduce blood pressure[2]. The mechanisms by which *n*-3 PUFA interact with the risk of MI in the present study are uncertain, but the analyses suggest that the content of marine *n*-3 PUFA is important, as the inverse relationship between fish intake and MI was observed for fatty fish and not lean fish. Furthermore, intake of fatty fish was highly correlated with intake of total and individual *n*-3 PUFA (EPA and DHA in particular). A few studies have examined the effect of individual marine *n*-3 PUFA, as most trials have

Table 3. Association of myocardial infarction and calculated intakes of individual and total marine *n*-3 PUFA*
(Hazard ratios (HR) and 95 % confidence intervals)

	Men								Women							
	Model A1†		Model A2‡		Model B§		Model Cǁǁǁǁǁǁǁǁ		Model A1†		Model A2‡		Model B§		Model Cǁǁǁǁǁǁǁǁ	
	HR	95 % CI	HR	95 % CI	HR	95 % CI	HR	95 % CI	HR	95 % CI	HR	95 % CI	HR	95 % CI	HR	95 % CI
EPA									EPA							
Q1 (0–99 mg)	1		1		1		1		Q1 (0–82 mg)	1		1		1		
Q2 (>99–151 mg)	0·88	0·77, 1·01	0·90	0·79, 1·03	0·89	0·78, 1·02	0·90	0·79, 1·03	Q2 (>82–125 mg)	0·93	0·76, 1·14	1·00	0·81, 1·22	0·98	0·80, 1·21	1·01	0·82, 1·24
Q3 (>151–207 mg)	0·93	0·82, 1·06	0·98	0·86, 1·12	0·97	0·85, 1·11	0·97	0·85, 1·12	Q3 (>125–173 mg)	0·74	0·60, 0·92	0·82	0·66, 1·02	0·80	0·65, 1·00	0·84	0·67, 1·05
Q4 (>207–294 mg)	0·92	0·81, 1·05	0·99	0·87, 1·13	0·97	0·85, 1·11	0·98	0·86, 1·13	Q4 (>173–247 mg)	1·00	0·82, 1·22	1·14	0·93, 1·39	1·11	0·91, 1·35	1·18	0·96, 1·46
Q5 (>294 mg)	0·89	0·78, 1·01	0·93	0·81, 1·07	0·90	0·79, 1·03	0·92	0·80, 1·07	Q5 (>247 mg)	0·75	0·61, 0·92	0·83	0·67, 1·03	0·79	0·64, 0·97	0·87	0·69, 1·10
P_{trend}	$P=0·20$		$P=0·76$		$P=0·42$		$P=0·69$		P_{trend}	$P=0·04$		$P=0·36$		$P=0·14$		$P=0·73$	
DPA									DPA							
Q1 (0–58 mg)	1		1		1		1		Q1 (0–42 mg)	1		1		1		
Q2 (>58–74 mg)	0·87	0·76, 1·00	0·88	0·77, 1·01	0·88	0·77, 1·01	0·87	0·75, 1·00	Q2 (>42–56 mg)	0·79	0·64, 0·97	0·83	0·68, 1·02	0·83	0·67, 1·02	0·84	0·68, 1·04
Q3 (>74–91 mg)	0·98	0·86, 1·12	0·98	0·86, 1·12	0·99	0·87, 1·13	0·97	0·84, 1·12	Q3 (>56–69 mg)	0·77	0·63, 0·94	0·84	0·68, 1·03	0·83	0·67, 1·02	0·86	0·69, 1·07
Q4 (>91–113 mg)	0·98	0·86, 1·12	0·98	0·85, 1·12	0·98	0·85, 1·11	0·96	0·82, 1·11	Q4 (>69–88 mg)	0·87	0·71, 1·06	0·92	0·76, 1·13	0·91	0·74, 1·11	0·95	0·76, 1·20
Q5 (>113 mg)	0·97	0·85, 1·11	0·94	0·82, 1·07	0·94	0·82, 1·07	0·92	0·78, 1·08	Q5 (>88 mg)	0·80	0·65, 0·98	0·82	0·67, 1·00	0·81	0·66, 0·99	0·84	0·66, 1·08
P_{trend}	$P=0·78$		$P=0·84$		$P=0·61$		$P=0·74$		P_{trend}	$P=0·11$		$P=0·19$		$P=0·13$		$P=0·46$	
DHA									DHA							
Q1 (0–257 mg)	1		1		1		1		Q1 (0–211 mg)	1		1		1		
Q2 (>257–373 mg)	0·91	0·80, 1·05	0·93	0·81, 1·06	0·93	0·81, 1·06	0·92	0·80, 1·05	Q2 (>211–307 mg)	0·81	0·66, 0·99	0·86	0·70, 1·06	0·87	0·71, 1·07	0·87	0·71, 1·08
Q3 (>373–496 mg)	0·92	0·80, 1·05	0·95	0·83, 1·08	0·95	0·83, 1·09	0·94	0·82, 1·07	Q3 (>307–413 mg)	0·79	0·64, 0·96	0·87	0·70, 1·07	0·86	0·70, 1·05	0·89	0·72, 1·10
Q4 (>496–683 mg)	0·96	0·84, 1·10	1·01	0·88, 1·15	1·00	0·87, 1·14	0·99	0·86, 1·14	Q4 (>413–573 mg)	0·88	0·72, 1·07	0·97	0·79, 1·18	0·95	0·78, 1·16	0·99	0·80, 1·23
Q5 (>683 mg)	0·90	0·79, 1·03	0·92	0·80, 1·05	0·90	0·78, 1·03	0·90	0·77, 1·05	Q5 (>573 mg)	0·75	0·61, 0·93	0·82	0·66, 1·01	0·79	0·64, 0·97	0·85	0·67, 1·07
P_{trend}	$P=0·29$		$P=0·56$		$P=0·34$		$P=0·44$		P_{trend}	$P=0·04$		$P=0·22$		$P=0·09$		$P=0·49$	
Total marine *n*-3 PUFA									Total marine *n*-3 PUFA							
Q1 (0–421 mg)	1		1		1		1		Q1 (0–341 mg)	1		1		1		
Q2 (>421–602 mg)	0·94	0·82, 1·07	0·95	0·83, 1·09	0·95	0·83, 1·08	0·94	0·82, 1·08	Q2 (>341–491 mg)	0·84	0·68, 1·03	0·90	0·74, 1·11	0·91	0·74, 1·11	0·92	0·75, 1·13
Q3 (>602–793 mg)	0·92	0·80, 1·05	0·95	0·83, 1·09	0·96	0·84, 1·09	0·94	0·82, 1·08	Q3 (>491–656 mg)	0·78	0·63, 0·96	0·86	0·70, 1·06	0·85	0·69, 1·04	0·88	0·71, 1·10
Q4 (>793–1083 mg)	0·95	0·83, 1·09	1·00	0·87, 1·14	0·99	0·86, 1·13	0·98	0·85, 1·13	Q4 (>656–904 mg)	0·88	0·72, 1·07	0·97	0·80, 1·19	0·95	0·78, 1·17	1·01	0·81, 1·25
Q5 (>1083 mg)	0·92	0·80, 1·05	0·94	0·82, 1·07	0·91	0·80, 1·04	0·92	0·79, 1·07	Q5 (>904 mg)	0·77	0·63, 0·95	0·84	0·69, 1·04	0·81	0·66, 0·99	0·88	0·69, 1·11
P_{trend}	$P=0·29$		$P=0·60$		$P=0·36$		$P=0·49$		P_{trend}	$P=0·04$		$P=0·25$		$P=0·11$		$P=0·56$	

Q, quintiles; DPA, docosapentaenoic acid.
* *P* values test the hypothesis of no trend across quintiles.
† Model A1: crude analysis.
‡ Model A2: adjusted for traditional risk factors including smoking, BMI, waist circumference, physical activity, alcohol intake, educational level and menopausal status (women).
§ Model B: adjusted as model A2 with additional covariates: history of diabetes mellitus, hypertension and hypercholesterolaemia.
ǁ Model C: adjusted as model A2 with additional dietary covariates: total energy intake, total intake of SFA, MUFA and PUFA (excl. marine *n*-3 PUFA) and dietary fibre.

Table 4. Association of dietary fish consumption and intake of marine *n*-3 PUFA with fatal myocardial infarction*
(Hazard ratios (HR) and 95 % confidence intervals)

	Men							
	Model A1†		Model A2‡		Model B§		Model C‖	
	HR	95 % CI	HR	95 % CI	HR	95 % CI	HR	95 % CI
Intake of fatty fish								
Q1 (0–8 g)	1		1		1		1	
Q2 (>8–13 g)	0·75	0·56, 1·01	0·77	0·57, 1·04	0·76	0·57, 1·03	0·79	0·59, 1·07
Q3 (>13–18 g)	0·74	0·55, 1·00	0·80	0·59, 1·06	0·80	0·60, 1·08	0·82	0·60, 1·10
Q4 (>18–28 g)	0·76	0·57, 1·02	0·85	0·63, 1·14	0·85	0·63, 1·14	0·88	0·65, 1·19
Q5 (>28 g)	0·78	0·58, 1·04	0·86	0·64, 1·15	0·81	0·61, 1·09	0·89	0·65, 1·21
P_{trend}	$P = 0·13$		$P = 0·50$		$P = 0·33$		$P = 0·66$	
Intake of lean fish								
Q1 (0–8 g)	1		1		1		1	
Q2 (>8–13 g)	0·90	0·65, 1·24	0·95	0·69, 1·31	0·97	0·70, 1·34	0·99	0·72, 1·37
Q3 (>13–18 g)	1·11	0·82, 1·50	1·17	0·87, 1·59	1·16	0·86, 1·58	1·26	0·92, 1·71
Q4 (>18–28 g)	1·09	0·80, 1·48	1·18	0·87, 1·60	1·17	0·86, 1·58	1·27	0·93, 1·74
Q5 (>28 g)	1·19	0·88, 1·60	1·23	0·91, 1·66	1·18	0·88, 1·60	1·34	0·97, 1·84
P_{trend}	$P = 0·12$		$P = 0·07$		$P = 0·14$		$P = 0·03$	
Total marine *n*-3 PUFA intake								
Q1 (0–421 mg)	1		1		1		1	
Q2 (>421–602 mg)	0·76	0·55, 1·04	0·77	0·56, 1·05	0·75	0·55, 1·03	0·76	0·56, 1·05
Q3 (>602–793 mg)	0·96	0·72, 0·29	1·00	0·74, 1·34	1·01	0·75, 1·35	1·00	0·73, 1·35
Q4 (>793–1083 mg)	0·93	0·69, 1·25	0·98	0·73, 1·32	0·96	0·72, 1·30	0·99	0·72, 1·35
Q5 (>1083 mg)	0·94	0·70, 1·26	0·95	0·71, 1·28	0·90	0·67, 1·21	0·99	0·71, 1·37
P_{trend}	$P = 0·86$		$P = 0·72$		$P = 0·97$		$P = 0·58$	

	Women							
	Model A1†		Model A2‡		Model B§		Model C‖	
	HR	95 % CI	HR	95 % CI	HR	95 % CI	HR	95 % CI
Intake of fatty fish								
Q1 (0–6 g)	1		1		1		1	
Q2 (>6–10 g)	1·04	0·66, 1·63	1·15	0·73, 1·82	1·15	0·73, 1·81	1·18	0·75, 1·87
Q3 (>10–15 g)	0·92	0·58, 1·46	1·06	0·67, 1·70	1·04	0·65, 1·65	1·10	0·69, 1·78
Q4 (>15–23 g)	0·49	0·28, 0·85	0·60	0·34, 1·05	0·58	0·33, 1·02	0·62	0·35, 1·09
Q5 (>23 g)	0·75	0·47, 1·21	0·94	0·58, 1·52	0·87	0·53, 1·40	0·94	0·56, 1·56
P_{trend}	$P = 0·03$		$P = 0·22$		$P = 0·12$		$P = 0·25$	
Intake of lean fish								
Q1 (0–6 g)	1		1		1		1	
Q2 (>6–10 g)	0·78	0·47, 1·31	0·85	0·51, 1·42	0·86	0·51, 1·44	0·89	0·53, 1·50
Q3 (>10–15 g)	1·07	0·67, 1·71	1·18	0·74, 1·89	1·17	0·73, 1·88	1·25	0·77, 2·03
Q4 (>15–23 g)	0·91	0·56, 1·48	1·04	0·64, 1·70	1·03	0·63, 1·68	1·12	0·67, 1·86
Q5 (>23 g)	0·95	0·59, 1·54	1·05	0·65, 1·70	1·03	0·64, 1·68	1·12	0·66, 1·90
P_{trend}	$P = 0·95$		$P = 0·62$		$P = 0·70$		$P = 0·46$	
Total marine *n*-3 PUFA intake								
Q1 (0–341 mg)	1		1		1		1	
Q2 (>341–491 mg)	1·09	0·69, 1·72	1·20	0·76, 1·90	1·19	0·75, 1·89	1·25	0·78, 2·00
Q3 (>491–656 mg)	0·82	0·50, 1·33	0·92	0·56, 1·50	0·90	0·55, 1·47	0·94	0·56, 1·57
Q4 (>656–904 mg)	0·75	0·45, 1·23	0·86	0·52, 1·43	0·84	0·51, 1·39	0·90	0·52, 1·54
Q5 (>904 mg)	0·75	0·46, 1·23	0·85	0·52, 1·39	0·81	0·49, 1·33	0·79	0·44, 1·41
P_{trend}	$P = 0·09$		$P = 0·24$		$P = 0·17$		$P = 0·22$	

Q, quintiles.
* *P* values test the hypothesis of no trend across quintiles.
† Model A1: crude analysis.
‡ Model A2: adjusted for smoking, BMI, waist circumference, physical activity, alcohol intake, educational level and menopausal status (women).
§ Model B: adjusted as model A2 with additional covariates: history of diabetes mellitus, hypercholesterolaemia and hypertension.
‖ Model C: Adjusted as model A2 with additional covariates. For dietary intake of fish, we adjusted for dietary covariates: total energy intake, intake of fruits and vegetables and intake of nuts. For intake of total *n*-3 PUFA, we adjusted for specific nutrients: total intake/content of SFA, MUFA and PUFA (excl. marine *n*-3 PUFA) and dietary fibre. Total marine *n*-3 PUFA was adjusted for total energy intake.

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examined an intervention consisting of a combination of EPA and DHA, as these are the major marine *n*-3 PUFA found in seafood and fish oil. However, a large intervention trial in a Japanese population used a supplement of purified EPA and produced a significant reduction in major cardiovascular events, suggesting anti-atherosclerotic effects of EPA independently of other marine *n*-3 PUFA[19]. Accordingly, important differential effects have been suggested between the major *n*-3 PUFA, but more studies on individual *n*-3 PUFA are warranted[40]. In the present study, we showed that EPA, DPA and DHA were similarly related to MI, but the study design did not allow for further investigation into the mechanistic aspects of individual *n*-3 PUFA.

Strengths and limitations

This study was based on a large prospective cohort study and holds the advantages of the prospective design.

The dietary intake of fish and marine *n*-3 PUFA was evaluated in a detailed manner. First, we assessed fish intake using detailed semi-quantitative FFQ including twenty-six questions concerning fish intake. This allowed us to differentiate by types of fish, thereby grouping fish consumption into fatty and lean fish depending on the content of marine *n*-3 PUFA. Second, we calculated the intake of major marine *n*-3 PUFA (EPA, DPA and DHA), to assess these fatty acids individually.

There was a limited loss to follow-up, and the assessment of outcome data was thorough and complete. All outcome data from the National Patient Registry or Causes of Death Registry were either examined from medical records or from a complete list of diagnoses and medical procedure codes, ensuring high validity of cases.

Adjustment for potential confounding was applied in different steps allowing for a more detailed interpretation of the results. In model A2, we adjusted for traditional risk factors for MI, which has a clear and straightforward interpretation. In model B, we applied a second layer of variables adjusting for medical history. These variables are normally considered traditional risk factors for MI and potential confounders, but at the same time may represent intermediate steps in causal pathways by which *n*-3 PUFA affect the risk of MI. In our opinion, model B represents the most complete analysis including important covariates, and the risk of introducing bias by including intermediate variables is outweighed by the risk of residual confounding by not including these variables. This is, however, open for discussion. Finally, we included dietary variables in addition to the traditional risk factors from model A2 (model C). This model implies adjustment for potential confounding from other aspects of the diet, but it may also introduce dietary patterns that are less comparable with the ordinary dietary pattern. Generally, the differences in measures of association between models were moderate or small, suggesting that confounding was not a major concern in this study.

This study also has certain limitations. Although age was evenly distributed among men and women, there was a markedly higher proportion of male cases and there were relatively few female cases, which made the CI wider for measures of association when analysing data in women, particularly fatal cases. The median follow-up period was 17·0 years, and dietary measures were not assessed during the study period. A long follow-up period allowed us to accumulate more outcome events, but the participants might have changed their diet over time. Furthermore, changes in standard medical care and general changes in lifestyle and public awareness of disease prevention might have influenced the participants' risk profile. To address this issue, we performed supplementary analysis by stratifying date of birth and testing for interaction from date of birth. No significant interaction was detected, and stratification did not affect the measures of association. Another concern when using FFQ is the chance of measurement error and various sources of bias, and evaluating biomarkers of marine *n*-3 PUFA intake would add strength to the dietary data. In this article we do not present data on biomarkers of *n*-3 PUFA, but our group recently published an article investigating the association between adipose tissue content of marine *n*-3 PUFA and MI, supporting the findings of this dietary study[41].

Conclusions

In this prospective cohort study, we found a high intake of fatty fish to be inversely related to incident MI in both men and women when comparing the highest and lowest quintiles. However, a clear dose–response relationship could not be established, and the test for trends across quintiles was not statistically significant in the adjusted analyses. Lean fish was not associated with MI. This study supports the current view that consumption of fatty fish may protect against MI.

Acknowledgements

This study was supported by a research grant from The Danish Heart Association, Copenhagen, Denmark, and the Hertha Christensen Foundation, Aalborg, Denmark. The Diet, Cancer and Health study was funded by the Danish Cancer Society. The sponsors neither had influence on the design and conduct of the study nor the writing of the manuscript.

All the authors contributed to the conception and planning of the study. A. G. and S. L.-C. were responsible for the statistical analysis of the data, and all authors were involved in interpretation of the data and writing of the manuscript. A. G. wrote the first draft of the manuscript.

The authors declare that there are no conflicts of interest regarding the present study.

Supplementary material

For supplementary material/s referred to in this article, please visit http://dx.doi.org/10.1017/S000711451600180X

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Appendix B. Paper II

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Adipose Tissue Content of Marine N-3 Polyunsaturated Fatty Acids Is Inversely Associated With Myocardial Infarction

Several, but not all, cohort studies have reported an inverse relationship between intake of marine n-3 polyunsaturated fatty acids (PUFA) and coronary heart disease (CHD) (1). Most epidemiological studies have assessed the intake of n-3 PUFA using questionnaires, but interestingly, biomarker studies have more consistently reported an inverse association of n-3 PUFA with CHD. In particular, adipose tissue marine n-3 PUFA has been suggested as an appropriate biomarker, as it reflects intake and metabolism during the past 1 to 2 years (1).

The Danish Diet, Cancer, and Health study is a cohort study previously described in detail (2). Briefly, 57,053 subjects between 50 and 65 years of age were enrolled between 1993 and 1997 and followed until July 2013. For this study, we used a case-cohort design including cases of myocardial infarction (MI) and a randomly selected subcohort (n = 3,500). We identified all participants in the cohort registered with an incident diagnosis of MI in the Danish National Patient Registry and/or the Danish Causes of Death Registry, according to the International Classification of Disease coding. Furthermore, cases of cardiac arrest were included if they were considered to be of cardiac origin after validation. At baseline, each participant filled in a detailed questionnaire on diet, life-style, and medical history. Blood samples were drawn, and an adipose tissue biopsy was collected from the buttocks, as previously described (3). The composition of fatty acids in adipose tissue was determined by gas chromatography, and results were expressed as weight percent of total fatty acids (3). Measures of association were assessed using Cox proportional hazards models with age as the time axis. We used a weighting scheme to account for the size of the subcohort. Potential confounders were selected a priori, and the proportional hazards assumption was checked, with no significant violations. Data were analyzed using STATA version 13.1 (StataCorp LP, College Station, Texas). The study was conducted according to the Helsinki Declaration and was approved by the regional ethics committees.

We identified 3,089 cases of incident MI during a median follow-up of 17 years. The subcohort resembled the entire cohort on all baseline parameters. We excluded subjects for whom information regarding covariates used in the adjusted analyses was missing (n = 643) and a further 421 subjects (214 cases and 207 subcohort) because of missing data regarding adipose tissue composition, leaving 2,814 cases and 3,163 participants from the subcohort for evaluation.

The associations of marine n-3 PUFA (eicosapentaenoic acid [EPA], docosahexaenoic acid [DHA] and docosapentaenoic acid [DPA]) in adipose tissue with incident MI are given in Table 1. Results for model A were adjusted for traditional risk factors for CHD and medical disorders, whereas model B included adjustment for traditional risk factors and dietary variables. We found an inverse association of adipose tissue content of EPA and DHA with incident MI. When comparing the highest and lowest quintile, hazard ratios of 0.76 (95% confidence interval: 0.63 to 0.91) and 0.78 (95% confidence interval: 0.64 to 0.95) were found for EPA and DHA, respectively. The associations were rather similar between models. Conversely, DPA was positively associated with MI

TABLE 1 Adipose Tissue Content of Marine n-3 PUFA and Association With Myocardial Infarction

	Model A*	Model B†
Content of EPA, %		
Q1 (0.00–0.07)	1.00	1.00
Q2 (>0.07–0.09)	0.98 (0.82–1.17)	0.93 (0.78–1.11)
Q3 (>0.09–0.11)	0.83 (0.69–1.00)	0.77 (0.63–0.93)
Q4 (>0.11–0.13)	0.92 (0.76–1.11)	0.84 (0.68–1.04)
Q5 (>0.13)	0.76 (0.63–0.91)	0.64 (0.50–0.82)
Content of DPA, %		
Q1 (0.00–0.21)	1.00	1.00
Q2 (>0.21–0.25)	1.21 (1.01–1.45)	1.26 (1.05–1.51)
Q3 (>0.25–0.29)	1.11 (0.91–1.36)	1.21 (0.97–1.50)
Q4 (>0.29–0.34)	1.11 (0.92–1.34)	1.26 (1.02–1.56)
Q5 (>0.34)	1.15 (0.94–1.40)	1.40 (1.08–1.80)
Content of DHA, %		
Q1 (0.00–0.18)	1.00	1.00
Q2 (>0.18–0.23)	0.93 (0.77–1.12)	0.91 (0.75–1.09)
Q3 (>0.23–0.29)	0.91 (0.75–1.09)	0.87 (0.71–1.05)
Q4 (>0.29–0.37)	0.97 (0.81–1.17)	0.88 (0.72–1.08)
Q5 (>0.37)	0.78 (0.64–0.95)	0.72 (0.56–0.94)

Values are hazard ratios (95% confidence intervals) from the Cox proportional hazards model. *Adjusted for traditional risk factors including: smoking, body mass index, waist circumference, physical activity, alcohol intake, educational level, and additionally, medical history of diabetes mellitus, hypertension, and hypercholesterolemia. †Adjusted for traditional risk factors and dietary variables: adipose tissue content of total saturated, monounsaturated and polyunsaturated (excluding marine n-3 PUFA) fatty acids and dietary fiber.

DHA = docosahexaenoic acid; DPA = docosapentaenoic acid; EPA = eicosapentaenoic acid; PUFA = polyunsaturated fatty acids; Q = quintile.

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when comparing the highest to the lowest quintile, although this was only statistically significant for model B. Pearson's correlations coefficients with dietary intake of the corresponding n-3 PUFA were modest for EPA ($r = 0.36$; $p < 0.001$) and DHA ($r = 0.34$; $p < 0.001$) and weak for DPA ($r = 0.08$; $p = 0.001$).

This study had limitations. The median follow-up was 17.0 years, and dietary measures were not assessed further during the study period. A long follow-up allowed us to accumulate more outcome events, but subjects might change their diet over time. Furthermore, changes in medical care and lifestyle together with public awareness of disease prevention might influence the participants' risk over time. To address this issue we performed supplementary analysis stratifying on the birthdate and tested for interaction from birthdate. No significant interaction was detected.

Several mechanisms have been suggested to explain cardioprotective properties of marine n-3 PUFA, including stabilization of atherosclerotic plaques, which may decrease the risk of plaque rupture and MI (1). The mechanism(s) by which n-3 PUFA interact with the risk of MI in the present study are uncertain, but the results suggest that EPA and DHA play a more important role than DPA. In conclusion, the study supports the view that marine n-3 PUFA may protect against MI.

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http://dx.doi.org/10.1016/j.jacc.2015.12.014

Please note: The study was supported by a research grant from The Danish Heart Association and Hertha Christensens Foundation. The Diet, Cancer and Health study was funded by the Danish Cancer Society. The sponsors had influence neither on the design and conduct of the study nor on the writing of the manuscript. The authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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Appendix C. Paper III

Title page

Title:

Common Polymorphisms in the 5-Lipoxygenase Pathway and Risk of Incident Myocardial Infarction: A Danish Case-Cohort Study

Short title:

Polymorphisms in the 5-Lipoxygenase Pathway and Risk of Myocardial Infarction

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Abstract

The 5-lipoxygenase pathway (5-LOX) has been implicated in the development of cardiovascular disease and studies have suggested that genetic polymorphisms related to key enzymes in this pathway may confer risk of myocardial infarction (MI). This study investigated the association of pre-selected genetic polymorphisms in four candidate genes of 5-LOX (arachidonate 5-lipoxygenase and its activating protein (*ALOX-5* and *FLAP*), leukotriene A4 hydroxylase (*LTA4-H*) and leukotriene C4 synthase (*LTC4-S*)) with incident MI.

In a Danish cohort including 57,053 participants, aged 50-64 at enrolment and recruited from 1993-97, we conducted a case-cohort study including cases with incident MI and a randomly selected sub cohort of 3,000 participants. Cases were identified from national registries through July 2013. A total of 22 SNPs were selected and genotyped using the commercially available KASP™ assay. A tandem-repeat polymorphism, located in the *ALOX-5* gene, was genotyped by multi-titre plate sequencing. Haplotypes were inferred using PHASE 2.1.

During a median follow-up of 17.0 years we identified 3,089 cases of incident MI. In *FLAP*, two SNPs were negatively associated with incident MI (rs9551963 & rs17222842) while one SNP (rs2247570) located in *LTA4-H*, was associated with higher risk of MI when comparing subjects with two copies of the variant allele to homozygotes for the wild type. Furthermore, the promoter polymorphism rs59439148 was associated with risk of MI in men. For male carriers of two variant alleles we found a hazard ratio of 1.63 (95% CI: 1.06;2.52) compared to homozygotes for the wild type. Previously described haplotypes (Hap-A -B, -E and –K) were not associated with MI in our population.

In conclusion, common polymorphisms in the 5-lipoxygenase pathway were associated with incident MI, suggesting a potential role for this pathway in the development of cardiovascular disease.

Introduction

Atherosclerosis is a multifactorial disease involving both environmental and genetic factors. In recent years, focus has turned on the complex cascade of inflammatory processes that takes place in the vessel wall and within atherosclerotic plaques [1,2]. In this context the 5-lipoxygenase (5-LOX) pathway has received attention. Thus, the 5-LOX pathway metabolizes arachidonic acid (AA), leading to the formation of highly pro-inflammatory lipid mediators called leukotrienes (LTs) [3]. The 5-LOX pathway consists of four key enzymes, where arachidonate 5-lipoxygenase (ALOX-5) and the ALOX-5 activating protein (FLAP) constitutes the first enzymatic step followed by conversion to either leukotriene C4 synthase (LTC4-S) or leukotriene B4 hydroxylase (LTB4-H)

resulting in the formation of either cysteinyl leukotrienes or B-series leukotrienes, respectively (Fig 1).

Fig 1. Schematic outline of the 5-LOX pathway leading to the formation of leukotrienes
The figure gives a schematic overview of the formation of 4-series leukotrines from arachidonic acid. The first step is catalysed by 5-lipoxygenase and 5-lipoxygenase activating protein (FLAP), which is also the rate-limiting step in the pathway. Next, leukotriene A4 is rapidly metabolised to either leukotriene B4 or the cysteinyl leukotrienes by leukotriene A4-hydroxylase or leukotriene C4-synthase, respectively.

Evidence at multiple levels, including animal, biochemical and human studies, has linked this pathway and the bioactive LTs to the development of atherosclerosis and athero-thrombotic disease [3–7]. Thus, in a mouse model, it was demonstrated that the knock out of the *ALOX-5* gene led to a high resistance against the development of atherosclerosis [8]. Other aspects of the 5-LOX pathway have been implicated in atherosclerosis traits in animal studies, including the leukotriene B4 – receptor [9,10] and the *FLAP* gene [11]. Furthermore, studies on human atherosclerotic plaques have identified the presence of 5-LOX enzymes and levels of ALOX-5 were higher in the more advanced plaques [12]. In addition, high levels of ALOX-5 and LTA4-H in human plaques have been associated with symptoms of plaque instability [13,14], suggesting a key role of the 5-LOX pathway in late stages of atherosclerosis and atherothrombotic events.

A number of epidemiological studies have examined four candidate genes, encoding the enzymes involved in the 5-LOX pathway. Most attention has been focused on *ALOX-5* and *FLAP*, where Dwyer et al. examined a tandem repeat polymorphism in the promoter region of *ALOX-5* that was associated with higher intima-media thickness of the carotid arteries, a marker of atherosclerosis, when comparing carriers of the variant allele with homozygotes of the wild type allele [15]. This polymorphism has been investigated in a number of studies with different endpoints, including ischemic stroke and MI [16–20], but the results have been conflicting.

In a genome-wide association study, the deCODE investigators identified *FLAP* as an important gene involved in atherothrombotic disease and reported two haplotypes (Hap-A and Hap-B) that were associated with higher risk of MI and stroke [21,22]. Some studies have supported these findings [23–27] while others did not find these haplotypes to be associated with risk of MI or stroke [19,28–30].

The deCODE investigators also defined a risk haplotype (Hap-K) for MI [31], covering the *LTA4-H* gene which was confirmed by other investigators [19,32]. Following these

studies, Zhao et al. defined a new haplotype (Hap-E) that was associated with a lower risk of MI among carriers compared to non-carriers [30].

Finally, two promoter polymorphisms have been identified in *LTC4-S* and investigated in three studies, with conflicting results [33–35].

Thus, from previous studies on four candidate genes, encoding key enzymes in the 5-LOX pathway, it has been suggested that genetic variants may be associated with atherosclerotic disease. Following a review of the literature, we selected 22 SNPs from these four candidate genes to investigate the association with incident MI in a large Danish cohort study.

Materials and Methods

Study design and population

The Danish Diet, Cancer and Health study is a prospective cohort study, which has been described in detail previously [36]. Briefly, 160,725 persons aged 50-64 years were invited between December 1993 and May 1997. Eligible participants were born in Denmark, living in the urban areas of Copenhagen and Aarhus and not registered with a cancer diagnosis in the Danish Cancer Registry at the time of invitation. If a cancer diagnosis was found that was not already recorded in the Cancer Registry at time of invitation, participants were excluded in line with the intention-to-include criteria. Further, participants registered with a previous MI or cardiac arrest were excluded as well. At baseline, each participant filled in a detailed questionnaire on diet, lifestyle, socio-economic status and medical history. Blood and adipose tissue samples were collected.

For the present study we used a nested case-cohort design including all cases with incident MI and a randomly selected sub cohort (n=3,000) to represent the cohort.
The study was conducted in accordance with the Helsinki Declaration and all participants provided written informed consent. The study, including the consent procedure, was approved by The Regional Ethics Committee, North Denmark Region (approval number, N-20140071).

Selection and genotyping of SNPs

From a review of the current literature, we selected four candidate genes to examine the 5-LOX pathway (*ALOX-5, FLAP, LTA4-H* and *LTC4-S*). Next, 22 SNP markers were selected based on previous reported associations with cardiovascular disease, with preference for coronary artery disease, and a confirmed minor allele frequency (MAF) > 0.05 in Caucasians.

From whole blood, DNA was extracted using Kleargene^{TM} XL DNA extraction kit (LGC Genomics, Queens Road, Teddington, Middlesex, UK). Next, contaminants were removed by washing and DNA was subsequently eluted into a low salt buffer. Extracted DNA were stored at -20°C.

SNP genotyping was performed by LGC Genomics using the commercially available KASP™ genotyping assay. KASP is based on a competitive, allele specific PCR genotyping technique with a homogenous fluorescent based reporting system [37]. The reaction mix was aliquoted to standard 96-well plates containing DNA-samples from the study cohort, including at least one "no template control" per plate. PCR was performed and the fluorescent signal was analysed using a BMG PHERAstar plate reader (BMG Labtech Ltd., Aylesbury, UK). The analysis was performed according to the protocol provided by LGC Genomics [38]. SNP alleles correspond to the positive/forward DNA-strand according to dbSNP, human assembly GRCh38.p2 [39].

Genotyping of *ALOX-5* tandem repeat polymorphism

The tandem repeat polymorphism was analysed by microtitre plate (MTP)-sequencing technique, using standard 96-well plates. PCR-products were prepared from genomic DNA, using MyTaq™ DNA polymerase (Bioline US Inc.) along with the following primers: 5'-TCAGGAGAGAACGAGTGAAC-3' (forward) and 5'-GTCCAGGTGTCCGCATC-3' (reverse). 40 reaction cycles were performed at 55°C. From the PCR-products, sequencing was done using an ABI 3730XL DNA analyser (Thermo Fischer Scientific Inc.) and Chromatograms were interpreted by a trained laboratory technician, identifying the number of tandem-repeats for each allele.

Outcome assessment

We identified all participants in the cohort who were registered with an incident diagnosis of MI in the Danish National Patient Registry and/or the Danish Causes of Death Registry, according to the International Classification of Disease (ICD) 8 (410.00-410.99) or ICD-10 (I21.0-I21.9) coding, during the study period. Furthermore, all cases of cardiac arrest (ICD-8: 427.27 or ICD-10: I46.0-I46.9) were included if the arrest was considered to be of cardiac origin after validation in each individual case. An earlier study from our Department validated the MI diagnosis from baseline through 2003 by complete review of all medical records and found a positive predictive value above 92 % when the diagnoses were obtained from a hospital ward [40]. All validated cases of MI from this validation study were included as cases for the present study. From January 2004 through July 2013 all participants with an incident MI diagnosed from a ward were readily accepted as cases without further validation. All other diagnoses of incident MI and cardiac arrest were validated by reviewing a complete list of diagnoses and interventional procedures recorded in the Danish National Patient Registry for each potential case.

Statistics

Allele frequencies were tested for Hardy–Weinberg equilibrium (HWE) in the sub cohort using a chi-square test (X^2-test). SNPs deviating from HWE ($p < 0.05$) were excluded from further analysis. We inferred haplotypes for combinations of SNPs using the program PHASE, version 2.1 [41,42]. In brief, the PHASE algorithm implements a Bayesian statistical method for reconstructing

haplotypes from observed genotype data, dealing with missing data by imputing missing genotypes. The program constructs diplotypes for each individual with probability estimates for each diplotype. Weights, from the probability estimates derived from PHASE, were implemented in the analyses as described by French et al. [43].

SNPs were analyzed as categorical variables with two degrees of freedom, assuming a general model of inheritance. To correct for multiple comparisons, we estimated the number of independent tests within each candidate gene, taking into account the correlation between SNPs by estimating the composite linkage disequilibrium (LD) correlation matrix from the SNP data, as described by Gao et al. [44]. Next, *p*-values were adjusted according to the number of independent tests derived from the correlation matrix using Sidák corrections [45]. Haplotype analyses were performed for previously described haplotypes in a univariate model, comparing the risk haplotype against all other haplotypes by including only the risk haplotype in the Cox model. Additionally, we evaluated all common haplotypes within *FLAP* and *LTA4-H* using a multivariate model. In this model, the most common haplotype was selected as reference and all common haplotype combinations (frequencies > 1%) derived from PHASE were compared to the common haplotype by including all haplotypes as covariates in the Cox model, except for the most common haplotype. For both the uni- and multivariate models, each haplotype was evaluated, comparing non-carriers with carriers of one or two copies of the haplotype, assuming linearity for the haplotype effect [43,46].

Measures of association were assessed using Cox proportional hazards multivariate regression models with age as the time axis and delayed entry. In accordance with the case-cohort design, we used a weighting scheme and robust variance estimates as described by Kalbfleisch and Lawless [47]. For the haplotype analyses, these weights were multiplied with the probability weights derived from PHASE. Analyses were conducted for the whole study cohort and separately for men and women, but the pooled analysis was considered as the primary. Participants were treated as at risk from baseline until either MI, death, emigration or end of follow-up occurred.

To address potential confounding we adjusted for traditional risk factors for MI (model A2) including smoking habits (never, former or current (<15 g/day, 15-25 g/day, >25 g/day) smoker), body mass index (kg/m^2), waist circumference (cm), physical activity (hours/week), alcohol intake (g/day), educational level (basic school, higher education: 1-3 years or >3 years) and, for women, menopausal status (pre- or post-menopausal). All continuous variables were included in the models as restricted cubic splines with five knots. Potential confounders were selected a priori based on current knowledge of risk factors for MI. In light of our primary exposure, being genetic polymorphisms, we did not expect confounding to be of major concern.

The proportional hazards assumption was checked by visual inspection of log-log plots and by evaluation of scaled Schoenfeld residuals with no violations of the assumption. P-values (two-tailed) < 0.05 were considered statistically significant. STATA, version 14.1 (StataCorp, College Station, TX, US) was used as statistical software.

Results

Population characteristics

From an initial 160,725 invited participants, a total of 57,053 (35%) accepted the invitation, and were enrolled into the study. From these, we excluded 1,506 participants due to missing baseline questionnaires or if recorded with a cancer diagnosis or having a previous MI or cardiac arrest before baseline. In our study population we identified 3,089 cases of incident MI during a median follow-up time of 17.0 years. After case verification, we excluded subjects for whom information regarding one or more potential confounders was missing. Additionally, 255 subjects were missing DNA-samples. In total, 2,876 cases were included in the analyses (Fig 2). For individual SNPs, genotype information was missing in 57 to 148 subjects.

As expected, traditional risk factors for MI were more prevalent among cases then in the sub cohort (Table 1).

Fig 2. Flow chart of cohort selection process

Table 1. Baseline characteristics of the sub cohort and cases.

	Men				Women			
	Sub cohort		Cases		Sub cohort		Cases	
Variable	(*n*=1,528)		(*n*=2,048)		(*n*=1,330)		(*n*=828)	
Age (years)	56.3	(51.2;63.3)	57.7	(51.7;63.9)	56.4	(51.1;63.0)	59.3	(52.4;64.2)
Physical activity (hours/week)	2.5	(0.0;8.5)	2.0	(0.0;8.0)	2.5	(0.0;8.0)	2.0	(0.0;7.0)
BMI (kg/m2)	26.4	(22.7;31.2)	26.9	(23.2;32.2)	24.6	(20.9;31.1)	25.9	(20.9;33.2)
Waist circumference (cm)	95.0	(85.0;109.0)	97.0	(86.0;112.0)	80.0	(69.0;97.0)	84.0	(70.0;102.0)
Alcohol intake (g/day)	19.4	(3.3;61.9)	18.2	(2.5;62.7)	9.3	(1.2;34.8)	6.5	(0.5;32.1)
Smoking (% (n))								
Never smoker	25.7	(392)	18.1	(370)	42.8	(569)	27.1	(224)
Former smoker	35.3	(540)	29.0	(594)	22.6	(301)	19.0	(157)
<15 g/day	11.1	(169)	12.7	(259)	16.2	(215)	22.7	(188)
15-25 g/day	16.8	(256)	24.2	(495)	15.6	(207)	26.1	(216)
>25 g/day	11.2	(171)	16.1	(330)	2.9	(38)	5.2	(43)
Educational level (% (n))								
Basic school	34.0	(520)	43.3	(887)	31.7	(422)	44.6	(369)
Higher education, 1-3 years	42.2	(645)	37.0	(758)	49.9	(664)	45.8	(379)
Higher education, >3 years	23.8	(363)	19.7	(403)	18.4	(244)	9.7	(80)
Menopausal status (% (n))								
Post-menopausal	-	-	-	-	59.6	(792)	69.9	(579)
Pre-menopausal	-	-	-	-	31.1	(413)	17.0	(141)
Medical history (% (n))								
Hypertension	14.9	(227)	22.2	(454)	17.1	(227)	31.3	(259)
Hypercholesterolaemia	8.4	(129)	12.0	(245)	6.5	(86)	13.0	(108)
Diabetes mellitus	3.1	(48)	5.4	(111)	1.4	(19)	4.5	(37)

Abbreviations: BMI, Body mass index.

Continuous variables are reported as medians (10th;90th percentile) and categorical variables as percent (n).

Association between *ALOX-5* tandem-repeat polymorphism and MI

A tandem-repeat polymorphism, located close to the promoter region of *ALOX-5*, was analyzed by traditional sequencing (rs59439148). The genotype frequencies, according to the number of hexamer-repeats ('-CCCGCC-') for the two alleles, are presented in Table 2. The 5-repeats allele was by far, the most common allele (84.4%). Next, the 4-repeats was the most frequent variant allele observed (15.2%). Alleles with less than 4-repeats were rare (<1%). As a result of the observed allele frequencies, we analyzed the tandem repeat defining the 5-repeats allele as the wild type and alleles with less than 5-repeats as the variant alleles. Frequencies of the constructed variant and wildtype are reported in Table 3. In men, the tandem-repeat polymorphism, rs59439148, was positively associated with MI when comparing homozygous carriers of the variant with carriers of the wild type (HR=1.63 with 95% CI: 1.06;2.52), suggesting a recessive genetic effect (S1 Table 4A). However, no association was seen for women and the combined analyses were not statistically significant (Table 4), although the hazard ratios suggested a positive association as in men.

In addition to the tandem-repeat polymorphism we also analyzed the SNP, rs12762303, that was previously shown to be in close LD with the variant and wild type allele of the tandem-repeat [29]. As anticipated, this SNP was in almost perfect LD with the tandem-repeat polymorphism (*D'*=0.99), and the measures of association were very similar to the tandem-repeat polymorphism.

Table 2. Distribution of genotypes for the ALOX-5 promoter polymorphism, according to number of tandem repeats (5'-GGGCGG-3').

Genotype	Sub cohort		Cases	
22	-	-	-	-
23	-	-	-	-
24	2	(0.07)	2	(0.07)
25	17	(0.59)	11	(0.38)
26	-	-	-	-
33	-	-	-	-
34	3	(0.10)	1	(0.03)
35	1	(0.03)	3	(0.10)
36	-	-	-	-
44	73	(2.55)	82	(2.85)
45	717	(25.09)	679	(23.61)
46	-	-	-	-
55	2,004	(70.12)	2,039	(70.90)
56	-	-	-	-
66	-	-	1	(0.03)

Abbreviations: ALOX-5, Arachidonate 5-lipoxygenase.

Reported as number of subjects with frequencies in parentheses (%). No observations are indicated with a dash.

Table 3. Minor allele frequency for each SNP, selected from four candidate genes in the 5-LOX pathway.

SNP	Genomic position	Allele	Sub cohort		Cases	
ALOX-5						
rs12762303	10: 45373723	C/T	14.9	(833)	14.4	(809)
rs59439148	10: 4537413(2-7)	V/W	15.8	(891)	15.3	(863)
FLAP						
rs17222814	13: 30725416	A/G	11.0	(619)	10.6	(599)
rs4073259	13: 30732134	G/A	35.9	(2,004)	35.3	(1,983)
rs10507391	13: 30737959	A/T	32.9	(1,855)	32.4	(1,836)
rs4769874	13: 30752304	A/G	3.8	(213)	3.6	(200)
rs9551963	13: 30758410	A/C	50.3	(2,831)	48.2	(2,723)
rs9315050	13: 30761908	G/A	6.1	(342)	6.5	(366)
rs17222842	13: 30765980	A/G	10.7	(598)	9.7	(547)
LTC4-S						
rs730012	5: 179793637	C/A	30.4	(1,705)	31.3	(1,765)
LTA4-H						
rs61937881	12: 95999809	T/C	24.3	(1,349)	25.7	(1,440)
rs2660880	12: 96007474	A/G	6.8	(385)	6.8	(387)
rs6538697	12: 96009832	C/T	7.2	(408)	7.4	(419)
rs1978331	12: 96015423	C/T	38.3	(2,144)	39.9	(2,249)
rs17677715	12: 96020673	C/T	17.7	(998)	19.0	(1,076)
rs2247570	12: 96028599	G/A	29.5	(1,653)	30.8	(1,735)
rs2660898	12: 96032219	G/T	31.8	(1,783)	33.1	(1,872)
rs2540482	12: 96041102	G/A	22.5	(1,263)	22.1	(1,251)
rs2540477	12: 96043776	C/T	22.0	(1,238)	21.7	(1,228)
rs2660845	12: 96044775	G/A	26.0	(1,460)	25.6	(1,447)
rs2540475	12: 96047515	T/C	20.5	(1,159)	20.0	(1,131)

Abbreviations: SNP, Single nucleotide polymorphism; ALOX-5, Arachidonate 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; LTC4-S, Leukotriene C4 synthase; LTA4-H, Leukotriene A4 hydroxylase.

Results presented as allele frequencies (n) for the minor allele(underlined). The two SNPs (rs17216473 & rs3776944) did not display variation in our study population. Alleles correspond to the positive DNA-strand and genomic position are obtained from dbSNP, human assembly GRCh38.p2.

Table 4. Association of selected single nucleotide polymorphisms with incident myocardial infarction.

SNP	Genotype	Model A1*		p^a	p^b	Model A2**		p^a	p^b
ALOX-5									
rs12762303	T/T	1 (ref)				1 (ref)			
	C/T	0.94	(0.83;1.07)	0.34	-	0.96	(0.84;1.10)	0.55	-
	C/C	1.27	(0.87;1.86)	0.22	-	1.37	(0.91;2.05)	0.13	-
rs59439148	W/W	1 (ref)				1 (ref)			
	W/V	0.94	(0.83;1.06)	0.31	-	0.96	(0.84;1.10)	0.52	-
	V/V	1.23	(0.89;1.71)	0.21	-	1.35	(0.96;1.90)	0.09	-
FLAP									
rs17222814	G/G	1 (ref)				1 (ref)			
	G/A	0.91	(0.79;1.05)	0.20	0.74	0.93	(0.80;1.08)	0.34	0.92
	A/A	1.22	(0.72;2.05)	0.47	0.98	1.18	(0.66;2.10)	0.58	0.99
rs4073259	A/A	1 (ref)				1 (ref)			
	A/G	0.95	(0.84;1.07)	0.38	0.94	0.93	(0.82;1.06)	0.28	0.86
	G/G	0.96	(0.81;1.15)	0.69	1.00	0.96	(0.80;1.17)	0.70	1.00
rs10507391	T/T	1 (ref)				1 (ref)			
	T/A	1.00	(0.89;1.12)	0.99	1.00	0.99	(0.87;1.12)	0.83	1.00
	A/A	0.94	(0.78;1.14)	0.55	0.99	0.98	(0.80;1.19)	0.82	1.00
rs4769874	G/G	1 (ref)				1 (ref)			
	G/A	0.96	(0.77;1.19)	0.72	1.00	1.04	(0.83;1.31)	0.74	1.00
	A/A	0.27	(0.06;1.28)	0.10	0.47	0.33	(0.07;1.61)	0.17	0.67
rs9551963	C/C	1 (ref)				1 (ref)			
	C/A	0.86	(0.75;0.98)	0.03	0.14	0.83	(0.72;0.96)	0.01	0.07
	A/A	0.85	(0.73;0.99)	0.04	0.21	0.80	(0.68;0.95)	0.01	0.05
rs9315050	A/A	1 (ref)				1 (ref)			
	A/G	1.03	(0.87;1.23)	0.71	1.00	1.07	(0.89;1.28)	0.50	0.98
	G/G	0.66	(0.28;1.55)	0.34	0.92	0.81	(0.34;1.93)	0.63	1.00
rs17222842	G/G	1 (ref)				1 (ref)			
	G/A	0.97	(0.85;1.12)	0.73	1.00	0.94	(0.81;1.10)	0.45	0.97
	A/A	0.40	(0.22;0.73)	0.01	0.02	0.44	(0.24;0.82)	0.01	0.05
LTC4-S									
rs730012	A/A	1 (ref)				1 (ref)			
	A/C	1.11	(0.99;1.25)	0.07	-	1.08	(0.95;1.22)	0.24	-
	C/C	1.02	(0.83;1.24)	0.88	-	1.00	(0.81;1.24)	0.99	-
LTA4-H									
rs61937881	C/C	1 (ref)				1 (ref)			
	C/T	1.05	(0.94;1.18)	0.40	0.95	1.02	(0.90;1.16)	0.71	1.00
	T/T	1.19	(0.94;1.50)	0.14	0.60	1.23	(0.96;1.58)	0.10	0.48
rs2660880	G/G	1 (ref)				1 (ref)			
	G/A	1.07	(0.91;1.27)	0.41	0.96	1.06	(0.89;1.28)	0.51	0.99
	A/A	0.83	(0.41;1.70)	0.61	1.00	0.80	(0.38;1.66)	0.54	0.99
rs6538697	T/T	1 (ref)				1 (ref)			
	T/C	1.01	(0.86;1.19)	0.88	1.00	1.04	(0.87;1.23)	0.69	1.00
	C/C	0.99	(0.45;2.21)	0.99	1.00	1.08	(0.48;2.43)	0.85	1.00
rs1978331	T/T	1 (ref)				1 (ref)			
	T/C	1.06	(0.94;1.19)	0.38	0.94	1.06	(0.93;1.21)	0.38	0.94
	C/C	1.15	(0.97;1.36)	0.10	0.48	1.19	(0.99;1.43)	0.06	0.30
rs17677715	T/T	1 (ref)				1 (ref)			
	T/C	1.05	(0.93;1.18)	0.43	0.97	1.04	(0.91;1.18)	0.56	0.99

	C/C	1.25	(0.91;1.72)	0.17	0.66	1.29	(0.92;1.81)	0.14	0.59
rs2247570	A/A	1 (ref)				1 (ref)			
	A/G	1.00	(0.90;1.13)	0.94	1.00	1.00	(0.88;1.13)	0.97	1.00
	G/G	1.23	(1.01;1.51)	0.04	0.22	1.28	(1.03;1.59)	0.03	0.15
rs2660898	T/T	1 (ref)				1 (ref)			
	T/G	1.09	(0.97;1.22)	0.17	0.66	1.07	(0.94;1.21)	0.32	0.90
	G/G	1.09	(0.90;1.32)	0.37	0.94	1.12	(0.92;1.38)	0.26	0.84
rs2540482	A/A	1 (ref)				1 (ref)			
	A/G	0.95	(0.85;1.07)	0.38	0.94	0.95	(0.84;1.08)	0.44	0.97
	G/G	1.05	(0.81;1.36)	0.74	1.00	0.98	(0.74;1.30)	0.89	1.00
rs2540477	T/T	1 (ref)				1 (ref)			
	T/C	0.95	(0.85;1.07)	0.39	0.95	0.95	(0.84;1.08)	0.45	0.97
	C/C	1.08	(0.83;1.41)	0.58	0.99	1.03	(0.77;1.37)	0.85	1.00
rs2660845	A/A	1 (ref)				1 (ref)			
	A/G	0.93	(0.83;1.05)	0.25	0.82	0.92	(0.82;1.04)	0.20	0.74
	G/G	1.04	(0.83;1.31)	0.74	1.00	1.04	(0.81;1.33)	0.76	1.00
rs2540475	C/C	1 (ref)				1 (ref)			
	C/T	1.00	(0.89;1.12)	0.97	1.00	0.98	(0.86;1.11)	0.72	1.00
	T/T	0.88	(0.67;1.17)	0.39	0.95	0.90	(0.67;1.21)	0.49	0.98

Abbreviations: SNP, Single nucleotide polymorphism; ALOX-5, Arachidonate 5-lipoxygenase; ALOX-5 AP, Arachidonate 5-lipoxygenase activating protein; LTC4-S, Leukotriene C4 synthase; LTA4-H, Leukotriene A4 hydroxylase; LD, linkage disequilibrium.

The table displays hazard ratios from a weighted cox proportional hazards model. Alleles correspond to the positive DNA-strand according to dbSNP, human assembly GRCh38.p2.

*Crude analyses. The pooled estimates are adjusted for sex.

**Adjusted analyses including sex(pooled analyses), smoking status, educational level, physical activity, BMI, waist circumference and alcohol consumption.

[a]Crude *p*-value.

[b]Adjusted *p*-value corrected for multiple testing within each candidate gene. From the composite LD correlation matrix the number of independent tests (*N*) were estimated. Using Sidák corrections, we then calculated the adjusted *p*-value as: $p^b = 1-(1-p^a)^N$.

Association of individual SNPs with MI

We genotyped 22 SNPs and examined associations with incident MI for each SNP individually. However, for two SNPs (rs17216473 and rs3776944), our assays were not able to detect the variant allele after testing two different assays on the forward strand and afterwards two assays on the reverse strand for each of the SNPs. The remaining 20 SNPs all displayed a MAF > 0.05 (Table 3) and the allele frequencies were similar to observations in other populations of European origin, according to dbSNP [39]. No SNPs deviated from the Hardy Weinberg equilibrium, when tested in the sub cohort.

In Table 4 we report hazard ratios for associations between individual SNPs and MI for the study cohort. Results are presented for both heterozygous and homozygous carriers of the variant allele compared to homozygous carriers of the wild type, assuming a general model of inheritance. Sex specific analysis are presented in the supplementary material (S1 Table 4A).

For *FLAP*, the SNP rs9551963 was negatively associated with MI when comparing both heterozygous and homozygous carriers of the minor allele (A) with homozygous carriers of the major allele (C), suggesting a dominant genetic effect. Results were similar across sex, but only significant in men. For the combined analyses, we found a HR of 0.80 (95% CI: 0.68;0.95) for homozygous carriers of the minor allele. Rs17222842 was also negatively associated with MI, when comparing homozygous carriers of the minor allele with homozygotes of the major (HR=0.28 (95% CI:0.12;0.63)) in men, while no association was observed in women.

Ten SNPs were successfully genotyped in *LTA4-H*. Rs2247570 was positively associated with MI in the combined analyses when comparing homozygous carriers of the minor (G) and major allele (A), HR=1.28 (95% CI: 1.03;1.59). This relationship was consistent in both men and women, but associations were not statistically significant in the sex-stratified analyses. For rs61937881, rs1978331 and rs17677715 we found a positive association between carriers of the minor allele and MI compared with carriers of the major allele in women. However, the associations were not consistent among men, and the associations were not significant in the combined analysis for men and women.

Finally, the SNP rs730012, located in proximity to the promoter region of *LTC4-S* was not associated with MI in our data.

Association of haplotypes with MI

Results from haplotype analyses are presented in Table 5 and 6. First, we tested single haplotypes, previously identified in other studies, using all remaining haplotypes as reference (Table 5). In the univariate analysis we did not find any of the previously described haplotypes to be associated with MI. Next, we performed multivariate analyses including all common haplotypes (haplotype frequency > 0.01) in the Cox-model, except for the most common haplotype, that served as reference (Table 6). When comparing carriers of each variant haplotype with carriers of the most common haplotype in *FLAP*, one haplotype was negatively associated with MI (GAGAAA). However, the association was modest. For *LTA4-H* the haplotype, CGTTTATAAT, was negatively associated with incident MI.

LD maps produced from the Haploview software showed that most, but not all the selected SNPs within *FLAP* and *LTA4-H*, were in high LD with one another. This raises the possibility that some degree of recombination within the haplotype blocks had occurred in our population (S2 Fig 3).

Table 5. Association of selected haplotypes in FLAP and LTA4-H with incident myocardial infarction.

Haplotype	Freq. (%)	Model A1*		p^a	Model A2**		p^a
FLAP							
Hap-A (GGAT)	14.0	0.97	(0.87;1.08)	0.58	0.92	(0.82;1.04)	0.18
Hap-B (AAG)	19.8	1.02	(0.93;1.12)	0.71	1.02	(0.92;1.13)	0.71
LTA4-H							
Hap-K (CGTTTATGGC)	14.5	0.94	(0.85;1.05)	0.28	0.91	(0.82;1.02)	0.12
Hap-E (CCTGAA)	7.6	1.05	(0.91;1.21)	0.49	1.08	(0.93;1.26)	0.33

Abbreviations: FLAP, 5-lipoxygenase activating protein; LTA4-H, Leukotriene A4 hydroxylase.

The table displays hazard ratios from a weighted cox proportional hazards model. Haplotypes were defined as follows: Hap-A (rs17222814(G), rs4769874(G), rs9551963(A), rs10507391(T)), Hap-B (rs10507391(A), rs9315050(A), rs17222842(G)), Hap-K (rs61937881(C), rs2660880(G), rs6538697(T), rs1978331(T), rs17677715(T), rs2247570(A), rs2660898(T), rs2540482(G), rs2660845(G), rs2540475(C)), Hap-E (rs61937881(C), rs1978331(C), rs17677715(T), rs2660898(G), rs2540482(A), rs2660845(A)). Alleles correspond to the positive DNA-strand according to dbSNP, human assembly GRCh38.p2.

*Crude analyses. The pooled estimates are adjusted for sex.

**Adjusted analyses including sex(pooled analyses), smoking status, educational level, physical activity, BMI, waist circumference and alcohol consumption.

[a]Unadjusted *p*-value.

Table 6. Multivariate test of haplotypes in FLAP and LTA4-H and association with incident myocardial infarction.

Haplotype	Freq. (%)	Model A1*		p^a	Model A2**		p^a
FLAP							
GTGCAG	40.6	1 (ref)			1 (ref)		
GAGAAG	16.1	0.95	(0.85;1.06)	0.35	0.94	(0.83;1.06)	0.29
GAGAAA	8.4	0.89	(0.77;1.02)	0.09	0.86	(0.74;0.99)	0.04
GAGCAG	3.7	1.19	(0.99;1.44)	0.07	1.18	(0.96;1.44)	0.12
GAACGG	3.5	0.88	(0.72;1.08)	0.23	0.96	(0.77;1.19)	0.71
GTGAAG	12.4	0.96	(0.85;1.08)	0.50	0.90	(0.79;1.02)	0.11
GTGAAA	1.7	0.82	(0.62;1.08)	0.17	0.85	(0.64;1.13)	0.26
GTGCGG	1.7	0.97	(0.73;1.29)	0.83	0.92	(0.67;1.26)	0.59
ATGAAG	10.6	0.92	(0.80;1.05)	0.21	0.92	(0.80;1.06)	0.27
LTA4-H							
CGTTTATAAC	43.7	1 (ref)			1 (ref)		
CGTTTATGGC	14.5	0.96	(0.86;1.07)	0.46	0.93	(0.82;1.05)	0.25
CGTTTATAAT	2.0	0.74	(0.57;0.96)	0.03	0.75	(0.56;0.99)	0.04
CGTCTGTAGC	3.1	1.03	(0.82;1.29)	0.81	1.03	(0.81;1.32)	0.80
CGTCTGTAAC	1.7	0.94	(0.70;1.26)	0.68	1.03	(0.76;1.40)	0.84

CGCCTAGAAC	5.5	1.04	(0.88;1.24)	0.63	1.04	(0.87;1.25)	0.65
CGCCTAGAAT	1.4	0.96	(0.70;1.31)	0.80	1.01	(0.73;1.39)	0.97
TGTCCGGGGC	6.2	1.12	(0.95;1.32)	0.19	1.16	(0.97;1.38)	0.10
TGTCCGGAAC	1.7	1.12	(0.86;1.47)	0.40	1.10	(0.83;1.45)	0.51
TGTCCGGAAT	9.7	1.05	(0.92;1.20)	0.49	1.03	(0.89;1.19)	0.69
TATCTGGAAT	5.5	1.00	(0.84;1.18)	1.00	1.00	(0.83;1.20)	0.99

Abbreviations: FLAP, 5-lipoxygenase activating protein; LTA4-H, Leukotriene A4 hydroxylase.

The table displays hazard ratios from a weighted cox proportional hazards model. All haplotypes with a frequency > 0.01 were included in the model except for the most common haplotype that represented the reference. Haplotypes were constructed from the following SNPs in order: rs17222814, rs10507391, rs4769874, rs9551963, rs9315050, rs17222842 (FLAP) and rs61937881, rs2660880, rs6538697, rs1978331, rs17677715, rs2247570, rs2660898, rs2540482, rs2660845, rs2540475 (LTA4-H). Alleles correspond to the positive DNA-strand according to dbSNP, human assembly GRCh38.p2.

*Crude analyses. The pooled estimates are adjusted for sex.

**Adjusted analyses including sex(pooled analyses), smoking status, educational level, physical activity, BMI, waist circumferrence and alcohol consumption.

[a]Unadjusted *p*-value.

Discussion

In the large Danish Diet, Cancer and Health cohort we undertook a case-cohort study, investigating the association between 20 pre-selected SNPs and incident MI. Single SNP analyses identified two markers in *FLAP* that were negatively associated with MI (rs9551963 and rs17222842), and for *LTA4-H*, rs2247570 was positively associated with MI. The tandem repeat polymorphism in *ALOX-5*, rs59439148, was positively associated with MI for men, while no association could be demonstrated in women. Furthermore, rs12762303 was in almost perfect LD with rs59439148. Finally, we performed haplotype analyses, first testing the association between previously reported haplotypes and MI with no significant associations found. Next, we tested haplotypes from *FLAP* and *LTA4-H* in multivariate analyses, including all inferred haplotypes with a frequency > 0.01, identifying one haplotype in each gene that was associated with MI.

In general, the associations of both single SNPs and haplotypes with MI were modest, and for some markers the results differed between men and women. A frequent concern in genetic association studies is the problem of multiple comparisons which raises the possibility of false positive discoveries (type I errors). We addressed this issue by limiting the number of SNP markers. Secondly, the number of statistical tests were minimized, e.g. for the single SNP analysis we tested the association with MI, assuming a general model of inheritance and refrained from testing several

specific models (e.g. dominant and recessive models). Some genetic association studies also adjust the significance threshold for multiple comparisons. A frequently used method is the Bonferroni correction which adjusts the significance level by the number of individual tests for each hypothesis. However, some authors have criticized this method for being too conservative, and thereby introducing false negative discoveries (type II errors) [48]. This is particularly of concern when testing multiple SNP-markers that are tightly correlated, and therefore not independent [44]. Accordingly, we used the method described by Gao et al. [44] to correct for multiple comparisons, taking into account the possible correlation between SNP-markers. We chose to report both the adjusted and the unadjusted *p*-values in Table 4. While, in our opinion, the unadjusted *p*-values represents the most clear interpretation of the data, they might be over optimistic and the results should be interpreted cautiously along with the following limitations and strengths to the study design. Further, all analyses were hypothesis driven and correction for multiple testing might not be of concern in this study.

Strengths and limitations

This study was based on a large prospective cohort study, and holds the advantages of the prospective design. There was a limited loss to follow-up and the assessment of outcome data was thorough and complete. The ethnicity of the study population was homogenous and all participants were of Caucasian ancestry. Furthermore, information on several dietary factors, lifestyle and other risk factors for MI was collected from the participants at baseline, allowing us to adjust for potential confounding. A priori, we did not expect confounding to be a major issue in this study, since the inherited genotype is subject to the principles of Mendelian randomization. However, some evidence suggests that epigenetics, including environmental factors, may influence on the expression of genes. We present the results for the crude model and an adjusted model (A2) including the most important risk factors for MI to address potential confounding. Furthermore, gender was not expected to biologically modify the associations between genotype and MI substantially, thus we considered the pooled analyses including both men and women to be the primary analyses. Secondary analyses, for men and women separately, were included as supplementary tables.

This study also had limitations. The selection of SNPs to cover the variation in each candidate gene was mostly based on findings by other studies and the results from genome wide scans, using a large number of genetic markers. While this method has proven to be highly effective in limiting the number of SNPs required for genotyping and the number of statistical tests to be performed, this method did not cover all common genetic variation within the candidate genes. Therefore, we cannot completely rule out that our study failed to capture all genetic variants that may be associated with our outcome. Two SNPs that were pre-selected could not be successfully

genotyped, and despite our efforts, four different assays were not able to detect the minor allele for these two SNPs (rs17216473 and rs3776944).

Even though age was evenly distributed among men and women there were few female cases, which made the confidence intervals wider for measures of association when analysing data in women, and in particular for rare polymorphisms. The median follow-up was 17.0 years, and dietary measures were not assessed during the study period. A long follow-up period allowed us to accumulate more outcome events, but subjects might change their lifestyle and habits over time. Furthermore, changes in standard medical care and public awareness of disease prevention might influence the participants' risk profile and limit our ability to address confounding. It is however unlikely that these changes affect measures of association for genotypes and confounding was a minor concern in this study.

General discussion

Since the initial findings by Dwyer et al. [15] concerning *ALOX-5*, and later the deCODE group identifying *FLAP* [21] and *LTA4-H* [31] as important genes in the 5-LOX pathway, a number of studies have been undertaken in effort to replicate and add evidence to these studies. The *ALOX-5* tandem repeat polymorphism (rs59439148) was first found to be associated with higher intima-media thickness by Dwyer et al. but replications on cases with MI and coronary artery disease verified by angiography, more clinically relevant endpoints, have yielded conflicting results. Notably, two independent case-control studies on MI patients with Northern European origin (Caucasians) did not support an association between variant alleles and MI for this polymorphism [17,18]. Another study in a mixed population of Caucasians and African-Americans demonstrated a positive association, only in African-Americans [19]. Generally, studies were small and no studies reported data on men and women separately. In the present study, we found a positive association between carriers of two variant alleles and MI, but interestingly, the association was only present among men while there was no association among women. The pooled analysis showed the same association as for men, but the test was not statistically significant. While we have no apparent biological explanation for the differences between men and women, these inconsistencies may either be explained by random variations in the small case-group (women) or by modification of the associations by gender. To our knowledge, no previous study has presented data for men and women separately and we have no data to compare to our own findings. Other SNPs have been explored in *ALOX-5* [19,29] but only rs59439148 and SNPs linked to this polymorphism has been associated with atherosclerosis traits. We confirmed the findings by Assimes et al. [29], that rs12762303 was in near perfect LD with rs59439148 and measures of association were similar for these two polymorphisms.

Looking at *FLAP*, Helgadottir et al. [21] identified two haplotypes, Hap-A and Hap-B, that was associated with risk of MI in carriers of Hap-A compared to non-carriers in an Icelandic population, while the same was true for Hap-B in a British population. The results were later replicated in a Scottish cohort confirming a positive association for Hap-A, but not Hap-B, with ischemic stroke [22]. In the present study, we could not confirm the association with MI for Hap-A or -B. However, we found two individual SNPs within Hap-A and -B to be associated with MI when comparing homozygous carriers of the minor allele vs. the major allele (rs9551963 and rs17222842). Other studies have found an association between individual SNPs and MI in these haplotype blocks, but no consensus in favor of a certain SNP has been agreed upon.

Finally, we analyzed ten SNPs covering a haplotype block in LTA4-H. Again, as for FLAP we found some of the individual SNPs to be associated with incident MI but when analyzing the previously reported haplotype (Hap-K) we did not find any association with MI in our population.

Additionally, we performed a global haplotype analyses for *FLAP* and *LTA4-H* SNPs, in order to explore new and unique haplotype combinations in our cohort. In these analyses we included all common haplotypes inferred to our cohort in a multivariate model using the most common haplotype as the reference haplotype. By this method we found sporadic associations between haplotypes and MI but the associations were modest. This method of analyzing haplotypes in a multivariate model is well established, but never the less, it has not been explored in previous studies on 5-LOX genes.

In the following decade since the deCODE studies, a number of large genome wide association studies (GWAS) exploring the role of common SNPs in MI has been conducted [49,50]. Notably, none of these studies have highlighted SNPs in the 5-LOX genes investigated in this study as important risk variants in MI. However, while these large scale studies have the advantage of covering the whole genome, they are not ideal in more specific hypothesis testing involving specific pathway genes. In this context, the 5-LOX pathway have been linked to atherosclerosis and MI in a variety of studies. The key mechanism linking the 5-LOX pathway to atherosclerosis lies in the formation of LT's and their bioactive properties. Notably, the expression of multiple 5-LOX enzymes has been linked to human atherosclerotic plaques and plaque development [12,13]. Furthermore, LT's are known to exert several pro-inflammatory effects including increased vascular permeability and chemo-attraction of monocytes [3,4]. Genetic association studies, mainly candidate gene studies, have also provided some evidence of a link between the 5-LOX pathway and atherosclerotic disease, which is supported by the present study. However, despite a number of studies performed it has not been possible to identify and confirm a functional polymorphism in most of the candidate genes, except for the tandem repeat polymorphism in *ALOX-5* (rs59439148),

and the studies performed have been heterogeneous regarding design and results. These inconsistencies have led some authors to propose a "pathway approach", taking into account that each step of the 5-LOX pathway may have a small influence on the outcome that can be detected when all steps are considered together. Crosslin et al. [32] examined how the expression of 5-LOX genes depended on the genotype of rs10507391, while another study found significant gene-gene interaction between a selected SNP from each of three 5-LOX genes [35]. Other studies have investigated the possibility that substrates of the 5-LOX pathway might interact with genetic variants in a complex environment where the effect of the genetic variant is dependent on substrate availability [16,51].

Conclusion

In this study we found single SNPs in three out of four candidate genes to be associated with incident MI, collectively suggesting that the 5-LOX pathway may play a role in MI. However, the associations were modest and some associations were not consistent for men and women separately. Association between MI and previously reported haplotypes, Hap-A, -B, -E and -K could not be confirmed.

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Supporting information

S1 Table 4A. Association of selected single nucleotide polymorphisms with incident myocardial infarction (sex-stratified analyses).

S2 Fig 3. Linkage disequilibrium (LD) plots.

Fig 1. Schematic outline of the 5-LOX pathway leading to the formation of leukotrienes

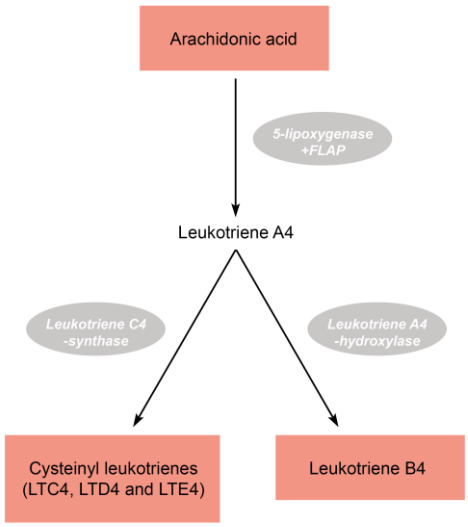
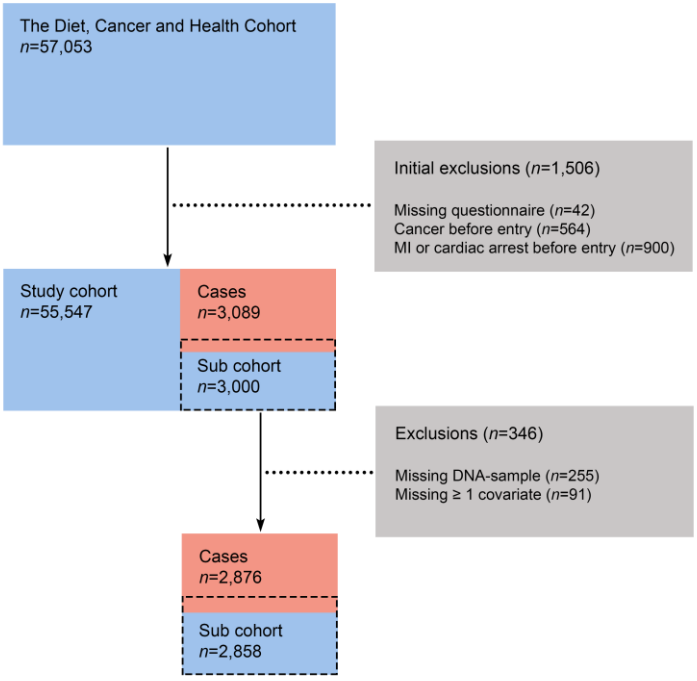


Fig 2. Flow chart of cohort selection process



S1 Table 4A. Association of selected single nucleotide polymorphisms with incident myocardial infarction (sex-stratified analyses).

		Men								Women							
SNP	**Genotype**	**Model A1***				**Model A2****				**Model A1***				**Model A2****			
				p^a	p^b			p^a	p^b			p^a	p^b			p^a	p^b
ALOX-5																	
rs12762303	T/T	1 (ref)				1 (ref)				1 (ref)				1 (ref)			
	C/T	0.99	(0.85;1.16)	0.90	-	1.00	(0.84;1.18)	1.00	-	0.83	(0.67;1.02)	0.08	-	0.83	(0.66;1.05)	0.12	-
	C/C	1.46	(0.89;2.39)	0.13	-	1.71	(1.03;2.84)	0.04	-	0.93	(0.51;1.71)	0.82	-	0.91	(0.45;1.85)	0.80	-
rs59439148	W/W	1 (ref)				1 (ref)				1 (ref)				1 (ref)			
	W/V	0.98	(0.84;1.15)	0.84	-	1.01	(0.85;1.19)	0.94	-	0.83	(0.67;1.03)	0.08	-	0.81	(0.65;1.03)	0.08	-
	V/V	1.45	(0.95;2.20)	0.09	-	1.63	(1.06;2.52)	0.03	-	0.86	(0.51;1.46)	0.59	-	0.91	(0.50;1.65)	0.75	-
FLAP																	
rs17222814	G/G	1 (ref)				1 (ref)				1 (ref)				1 (ref)			
	G/A	0.90	(0.75;1.06)	0.21	0.76	0.92	(0.77;1.10)	0.36	0.93	0.98	(0.78;1.23)	0.85	1.00	0.98	(0.76;1.26)	0.85	1.00
	A/A	1.24	(0.64;2.42)	0.52	0.99	1.14	(0.56;2.30)	0.72	1.00	1.26	(0.57;2.80)	0.57	0.99	1.34	(0.49;3.67)	0.57	0.99
rs4073259	A/A	1 (ref)				1 (ref)				1 (ref)				1 (ref)			
	A/G	0.96	(0.83;1.12)	0.63	1.00	0.94	(0.81;1.10)	0.48	0.98	0.90	(0.74;1.09)	0.28	0.86	0.87	(0.70;1.08)	0.21	0.76
	G/G	0.98	(0.79;1.21)	0.85	1.00	0.97	(0.78;1.22)	0.83	1.00	0.94	(0.70;1.26)	0.66	1.00	0.95	(0.68;1.33)	0.77	1.00
rs10507391	T/T	1 (ref)				1 (ref)				1 (ref)				1 (ref)			
	T/A	1.02	(0.89;1.18)	0.75	1.00	1.01	(0.86;1.17)	0.94	1.00	0.94	(0.78;1.14)	0.53	0.99	0.91	(0.73;1.12)	0.37	0.94
	A/A	0.97	(0.78;1.21)	0.79	1.00	1.01	(0.79;1.28)	0.95	1.00	0.90	(0.66;1.23)	0.52	0.99	0.93	(0.66;1.33)	0.70	1.00
rs4769874	G/G	1 (ref)				1 (ref)				1 (ref)				1 (ref)			
	G/A	0.97	(0.75;1.25)	0.80	1.00	1.03	(0.78;1.35)	0.86	1.00	0.95	(0.65;1.38)	0.77	1.00	1.02	(0.68;1.53)	0.94	1.00
	A/A	0.69	(0.10;4.76)	0.71	1.00	1.14	(0.16;8.06)	0.90	1.00	-	-	-	-	-	-	-	-
rs9551963	C/C	1 (ref)				1 (ref)				1 (ref)				1 (ref)			
	C/A	0.86	(0.73;1.02)	0.08	0.39	0.83	(0.70;0.99)	0.04	0.21	0.86	(0.69;1.06)	0.16	0.65	0.84	(0.65;1.07)	0.16	0.65
	A/A	0.83	(0.69;1.00)	0.06	0.29	0.78	(0.64;0.96)	0.02	0.09	0.92	(0.71;1.18)	0.50	0.98	0.86	(0.65;1.15)	0.31	0.89
rs9315050	A/A	1 (ref)				1 (ref)				1 (ref)				1 (ref)			
	A/G	1.01	(0.82;1.24)	0.94	1.00	1.04	(0.83;1.29)	0.74	1.00	1.10	(0.82;1.47)	0.52	0.99	1.17	(0.85;1.61)	0.34	0.92
	G/G	1.06	(0.36;3.10)	0.92	1.00	1.42	(0.47;4.32)	0.53	0.99	0.16	(0.02;1.28)	0.08	0.41	0.16	(0.02;1.45)	0.10	0.48
rs17222842	G/G	1 (ref)				1 (ref)				1 (ref)				1 (ref)			
	G/A	0.98	(0.82;1.17)	0.80	1.00	0.98	(0.81;1.18)	0.83	1.00	0.97	(0.77;1.22)	0.79	1.00	0.93	(0.72;1.20)	0.57	0.99
	A/A	0.27	(0.12;0.59)	0.00	0.01	0.28	(0.12;0.63)	0.00	0.01	1.00	(0.41;2.43)	0.99	1.00	1.01	(0.39;2.60)	0.99	1.00
LTC4-S																	
rs730012	A/A	1 (ref)				1 (ref)				1 (ref)				1 (ref)			
	A/C	1.12	(0.97;1.29)	0.11	-	1.11	(0.95;1.29)	0.19	-	1.09	(0.90;1.32)	0.37	-	1.00	(0.81;1.24)	1.00	-
	C/C	1.00	(0.78;1.27)	0.98	-	0.98	(0.76;1.27)	0.91	-	1.07	(0.78;1.48)	0.67	-	1.10	(0.76;1.59)	0.61	-
LTA4-H																	
rs61937881	C/C	1 (ref)				1 (ref)				1 (ref)				1 (ref)			
	C/T	0.99	(0.86;1.15)	0.93	1.00	0.96	(0.82;1.12)	0.59	1.00	1.21	(1.00;1.46)	0.05	0.26	1.25	(1.01;1.55)	0.04	0.21
	T/T	1.16	(0.88;1.55)	0.30	0.88	1.16	(0.86;1.58)	0.33	0.91	1.24	(0.85;1.81)	0.26	0.84	1.42	(0.95;2.13)	0.09	0.42

rs2660880	G/G	1 (ref)				1 (ref)				1 (ref)				1 (ref)					
	G/A	1.10	(0.89;1.35)	0.40	0.95	1.09	(0.87;1.37)	0.45	0.97	1.03	(0.79;1.34)	0.83	1.00	0.99	(0.72;1.34)	0.93	1.00
	A/A	0.57	(0.23;1.43)	0.23	0.80	0.53	(0.21;1.37)	0.19	0.72	1.53	(0.50;4.67)	0.45	0.97	1.56	(0.46;5.23)	0.48	0.98
rs6538697	T/T	1 (ref)				1 (ref)				1 (ref)				1 (ref)					
	T/C	0.97	(0.80;1.18)	0.76	1.00	1.00	(0.81;1.24)	0.98	1.00	1.12	(0.87;1.44)	0.40	0.95	1.04	(0.77;1.39)	0.82	1.00
	C/C	0.98	(0.39;2.45)	0.97	1.00	1.00	(0.39;2.57)	1.00	1.00	0.97	(0.21;4.53)	0.97	1.00	1.15	(0.23;5.80)	0.87	1.00
rs1978331	T/T	1 (ref)				1 (ref)				1 (ref)				1 (ref)					
	T/C	0.99	(0.86;1.15)	0.94	1.00	0.98	(0.83;1.14)	0.76	1.00	1.22	(1.00;1.49)	0.05	0.26	1.30	(1.04;1.62)	0.02	0.12
	C/C	1.12	(0.91;1.37)	0.30	0.88	1.14	(0.92;1.43)	0.24	0.80	1.24	(0.94;1.63)	0.13	0.56	1.27	(0.93;1.74)	0.13	0.56
rs17677715	T/T	1 (ref)				1 (ref)				1 (ref)				1 (ref)					
	T/C	1.00	(0.86;1.15)	0.95	1.00	0.96	(0.82;1.13)	0.62	1.00	1.19	(0.98;1.44)	0.09	0.42	1.32	(1.06;1.65)	0.01	0.08
	C/C	1.26	(0.85;1.86)	0.25	0.83	1.30	(0.86;1.98)	0.21	0.76	1.26	(0.75;2.12)	0.37	0.94	1.31	(0.76;2.24)	0.33	0.91
rs2247570	A/A	1 (ref)				1 (ref)				1 (ref)				1 (ref)					
	A/G	0.94	(0.82;1.09)	0.41	0.96	0.93	(0.80;1.08)	0.33	0.91	1.18	(0.98;1.43)	0.08	0.40	1.20	(0.97;1.48)	0.10	0.45
	G/G	1.28	(1.00;1.64)	0.05	0.28	1.28	(0.98;1.67)	0.07	0.35	1.11	(0.80;1.55)	0.54	0.99	1.27	(0.88;1.82)	0.20	0.74
rs2660898	T/T	1 (ref)				1 (ref)				1 (ref)				1 (ref)					
	T/G	1.04	(0.90;1.20)	0.57	0.99	1.02	(0.87;1.18)	0.84	1.00	1.19	(0.99;1.44)	0.07	0.35	1.22	(0.98;1.50)	0.07	0.35
	G/G	1.03	(0.81;1.30)	0.82	1.00	1.05	(0.82;1.34)	0.72	1.00	1.26	(0.93;1.72)	0.14	0.58	1.27	(0.89;1.82)	0.18	0.70
rs2540482	A/A	1 (ref)				1 (ref)				1 (ref)				1 (ref)					
	A/G	0.92	(0.80;1.07)	0.28	0.87	0.94	(0.80;1.09)	0.40	0.95	1.00	(0.83;1.21)	0.96	1.00	1.00	(0.81;1.23)	0.97	1.00
	G/G	1.10	(0.80;1.52)	0.55	0.99	1.04	(0.74;1.46)	0.84	1.00	0.88	(0.56;1.39)	0.59	1.00	0.83	(0.50;1.37)	0.46	0.98
rs2540477	T/T	1 (ref)				1 (ref)				1 (ref)				1 (ref)					
	T/C	0.93	(0.81;1.07)	0.32	0.90	0.95	(0.81;1.10)	0.48	0.98	0.99	(0.82;1.20)	0.95	1.00	0.96	(0.78;1.19)	0.73	1.00
	C/C	1.13	(0.81;1.56)	0.48	0.98	1.06	(0.75;1.50)	0.75	1.00	0.94	(0.60;1.48)	0.80	1.00	0.94	(0.57;1.56)	0.82	1.00
rs2660845	A/A	1 (ref)				1 (ref)				1 (ref)				1 (ref)					
	A/G	0.91	(0.79;1.04)	0.17	0.67	0.90	(0.78;1.05)	0.18	0.70	1.00	(0.83;1.21)	1.00	1.00	0.98	(0.79;1.20)	0.83	1.00
	G/G	1.05	(0.79;1.39)	0.72	1.00	1.04	(0.77;1.41)	0.78	1.00	0.98	(0.67;1.45)	0.94	1.00	1.00	(0.65;1.55)	1.00	1.00
rs2540475	C/C	1 (ref)				1 (ref)				1 (ref)				1 (ref)					
	C/T	0.96	(0.83;1.11)	0.58	0.99	0.93	(0.80;1.09)	0.39	0.95	1.11	(0.92;1.35)	0.28	0.86	1.12	(0.91;1.40)	0.29	0.87
	T/T	0.94	(0.66;1.33)	0.74	1.00	0.96	(0.67;1.38)	0.83	1.00	0.75	(0.47;1.20)	0.23	0.78	0.79	(0.47;1.33)	0.38	0.94

Abbreviations: SNP, Single nucleotide polymorphism; ALOX-5, Arachidonate 5-lipoxygenase; ALOX-5 AP, Arachidonate 5-lipoxygenase activating protein; LTC4-S, Leukotriene C4 synthase; LTA4-H, Leukotriene A4 hydroxylase.

The table displayes hazard ratios from a weighted cox proportional hazards model. Results are presented for sex-stratified analyses. Alleles correspond to the positive DNA-strand according to dbSNP, human assembly GRCh38.p2.

*Crude analyses. The pooled estimates are adjusted for sex.

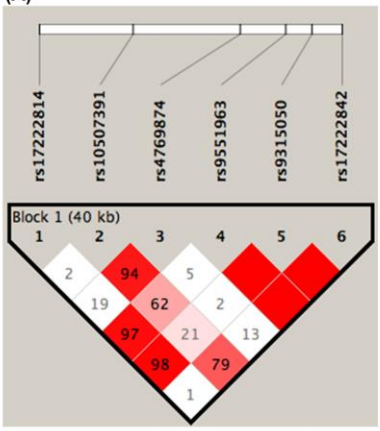
**Adjusted analyses including sex(pooled analyses), smoking status, educational level, physical activity, BMI, waist circumferrence and alcohol consumption.

[a]Crude *p*-value

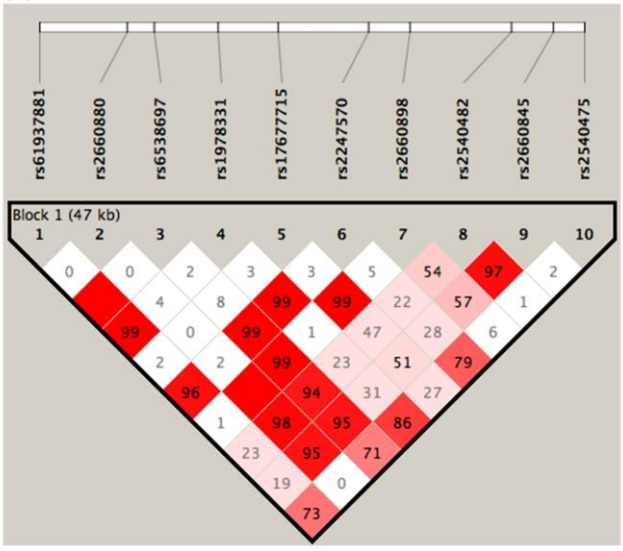
[b]Adjusted *p*-value corrected for multiple testing within each candidate gene. From the composite LD correlation matrix the number of independent tests (*N*) were estimated. Using Sidák corrections, we then calculated the adjusted *p*-value as: $p^b = 1-(1-p^a)^N$.

S2 Fig 3. Linkage disequilibrium (LD) plots

(A)



(B)



(A): LD plot for single nucleotide polymorphisms (SNP) spanning the *ALOX-5* gene. The plot reports values of D' (high values are indicated by high intensity colour).
(B): LD plot for SNP's spanning the *LTA4-H* gene.
LD plots were constructed using the Haploview software (BROAD Institute MIT, Cambridge, MA, USA).

Appendix D. Paper IV

1. Title page

Title

Diet-gene interaction between 5-lipoxygenase polymorphisms and substrates for the 5-lipoxygenase pathway modulates the risk of myocardial infarction - a Danish case-cohort study.

Authors

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2. Abstract

Background

Arachidonate 5-lipoxygenase (ALOX-5) is the rate limiting enzyme in the 5-lipoxygenase pathway that has been linked to atherothrombotic disease. A functional tandem repeat polymorphism in the *ALOX-5* gene has been associated with risk of myocardial infarction (MI). However, results from previous studies have been inconsistent, and interestingly, two studies reported an interaction between dietary intake of ALOX-5 substrates, arachidonic acid (AA) and eicosapentaenoic acid (EPA), and genotype.

Objective

We investigated the possible interaction between the *ALOX-5* tandem repeat polymorphism (rs59439148) and adipose tissue content of AA and EPA with incident MI.

Methods

In the Danish Diet, Cancer and Health study, we conducted a case-cohort study including 3,089 cases with incident MI identified from national registries and a randomly selected sub-cohort of 3,000 participants. Adipose tissue and blood samples were collected at baseline along with comprehensive questionnaires on lifestyle and demographic data. The *ALOX-5* tandem repeat polymorphism was genotyped by multi-titre plate sequencing.

Results

Cross tabulation of genotype by quintiles of EPA or AA content indicated a higher risk of MI for carriers of two variant alleles. Concerning AA, we observed the highest risk of MI for homozygous carriers of the variant in the highest quintile of AA content compared with the reference group with a low content of AA and carrying the wild type allele (HR=3.02, 95% CI: 1.41;6;44). In contrast, homozygotes for the variant had the highest risk of MI when comparing the lowest quintile of EPA content with the reference (HR=2.15, 95% CI: 0.91;5.09). Although our results suggested interaction, measures of association were not statistically significant.

Conclusions

In this large Danish cohort, homozygous carriers of the variant genotype had a higher risk of MI, and results indicated that the association was modulated by adipose tissue content of AA and EPA.

3. Introduction

Atherosclerosis is considered a multifactorial disorder involving complex inflammatory processes in the vessel wall [1,2]. In this context, the 5-lipoxygenase pathway (5-LOX) has received attention, and has been linked to atherothrombotic disease on multiple levels including both animal and human studies [3,4]. 5-LOX enzymes metabolise arachidonic acid (AA) and, to a lesser extent, eicosapentaenoic acid (EPA) leading to the formation of pro-inflammatory leukotrienes (LTs) (Figure 1). Importantly, the 5-series LTs derived from EPA are much less pro-inflammatory compared with 4-series LTs derived from AA [5], and studies have shown that consumption of marine n-3 polyunsaturated fatty acids (PUFA) (including EPA) can increase production of 5-series LTs at the expense of the more pro-inflammatory 4-series LTs [6]. Replacing AA with EPA may therefore elicit a relative anti-inflammatory effect.

In line with this, epidemiological studies have generally supported an inverse association between fish intake, including EPA, and risk of myocardial infarction (MI) [7,8]. In contrast, some studies have reported the intake of AA to be positively associated with the risk of MI [9]. We recently tested these findings in a Danish cohort study, the Diet, Cancer and Health study, confirming an inverse association of both dietary fatty fish [10] and adipose tissue content of EPA [11] with the risk of MI, while AA content in adipose tissue was positively associated with MI [12]. As a new approach, we used adipose tissue content of the respective fatty acids which is considered a good long-term marker of dietary intake of these fatty acids, and importantly, represents the endogenous exposure to these fatty acids [13,14].

A number of studies have examined genetic polymorphisms related to key enzymes in the 5-LOX pathway. Most attention has been focused on the rate-limiting step, catalysed by arachidonate 5-lipoxygenase (ALOX-5), where Dwyer et al. examined a tandem repeat polymorphism in the promoter region of *ALOX-5* (rs59439148), containing a varying number of SP1 transcription factor binding motifs (5'-GGGCGG-3'). The study found variant alleles associated with higher intima-media thickness of the carotid arteries compared with carriers of two wild type alleles [15]. Following the initial study, this polymorphism has been investigated in a number of studies with different endpoints, including ischemic stroke and MI [16–20], but results have been conflicting. Interestingly, some studies have suggested an interaction between genotype and intake of marine n-3 PUFA and/or AA [15,16], the substrates of the 5-LOX pathway. Thus, a high intake of marine n-3 PUFA seemed to blunt the effect of the variant genotype, while in contrast, a high intake of AA tended to exacerbate the effect of the variant genotype. Other studies have examined polymorphisms in the *ALOX-5* gene, but no polymorphisms, other than the tandem repeat has been confidently associated with CVD endpoints. Interestingly the SNP, rs12762303, was reported to be in close linkage disequilibrium (LD) with the variant and wild type alleles of the

tandem repeat [30], which could provide an easy and more cost-effective means of analysing the tandem repeat polymorphism.

Taken together, evidence suggests a complex interplay between polymorphisms in the 5-LOX system and dietary intake of AA and EPA. In this study, we examined the association of this *ALOX-5* tandem repeat polymorphism with incident MI, taking into account the AA and EPA levels in adipose tissue to investigate possible diet-gene interaction. Furthermore, we genotyped the SNP, rs12762303, that may prove to be in high linkage disequilibrium (LD) with the tandem repeat.

4. Methods

Study design and population

The Danish Diet, Cancer and Health study is a prospective cohort study, which has been described in detail previously [21]. Briefly, 160,725 persons aged 50-64 years were invited between December 1993 and May 1997. Eligible participants were born in Denmark, living in the urban areas of Copenhagen and Aarhus and not registered with a cancer diagnosis in the Danish Cancer Registry at the time of invitation. A total of 57,053 persons accepted the invitation and were enrolled into the study. Participants registered with a previous MI or cardiac arrest were excluded. If a cancer diagnosis was reported that was not already recorded in the Cancer Registry at the time of invitation, participants were excluded in line with the intention-to-include criteria. At baseline, each participant filled in a detailed questionnaire on diet, lifestyle, socio-economic status and medical history. Blood and adipose tissue samples were collected.

For the present study we used a nested case-cohort design including all cases with incident MI and a randomly selected sub-cohort (n=3,000) to represent the cohort.
The study was conducted in accordance with the Helsinki Declaration and approved by the regional ethics committees.

DNA extraction

DNA was extracted from whole blood using Kleargene^{TM} XL DNA extraction kit (LGC Genomics, Queens Road, Teddington, Middlesex, UK). The Kleargene method employs a detergent-driven cell lysis technique, followed by guanidinium isothiocyanate-mediated DNA binding to silica. Next, contaminants were removed by washing and DNA was subsequently eluted into a low salt buffer. The final DNA product was stored at -20°C until analysis.

Genotyping of *ALOX-5* tandem repeat polymorphism

The tandem repeat polymorphism was analysed by micro-titre plate (MTP)-sequencing technique, using standard 96-well plates. PCR products were prepared from genomic DNA, using MyTaq^{TM}

DNA polymerase (Bioline Inc., US) along with the following primers: 5'-TCAGGAGAGAACGAGTGAAC-3' (forward) and 5'-GTCCAGGTGTCCGCATC-3' (reverse). Forty reaction cycles were performed at 55°C. From the PCR products, sequencing was performed using an ABI 3730XL DNA analyser (Thermo Fischer Scientific Inc., US) and chromatograms were interpreted by a laboratory technician, identifying the number of tandem repeats for each allele. In case of unclear calls, the chromatograms were rechecked by another technician, and results were discussed and agreed upon.

Genotyping of rs12762303

SNP genotyping was performed by LGC Genomics using the commercially available KASP™ genotyping assay [22]. The fluorescent signal from the PCR products was analysed using a BMG PHERAstar plate reader (BMG Labtech Ltd., Aylesbury, UK). Analysis was performed according to the protocol provided by LGC Genomics [23]. SNP alleles correspond to the positive/forward DNA strand according to dbSNP, human assembly GRCh38.p2 [24].

Adipose tissue biopsies

At baseline, adipose tissue biopsies were taken from the buttocks of all participants using a luer lock system (Terumo, Terumo Corp, Tokyo, JP) consisting of a needle, a venoject multisample luer adaptor, and an evacuated blood tube, according to the method described by Beynen and Katan [25]. Samples were flushed with nitrogen and stored at -150°C until analysis. When analysed, biopsies were thawed and preheated at 50°C for 10 min. Subsequently, the fat was dissolved in heptane at 50°C, and fatty acids were transesterified by 2 mol/L KOH (potassium hydroxide) in methanol at 50°C for 2 minutes. The fatty acid composition was determined by gas chromatography using a Varian 3900 GC with a CP-8400 autosampler (Varian, Middleburg, NL) equipped with a flame ionisation detector. Split injection mode, a CP-sil 88, 50 m x 0.25 mm ID capillary column, temperature programming from 90°C to 210°C and constant flow were used. Helium was used as carrier gas. Commercially available standards (Nu-chek-Prep, Inc., Minnesota, US) were used to identify the individual fatty acids.

The content of fatty acids was expressed as weight percent of total fatty acids, and the inter assay coefficients of variation were 3.2% and 6.4% for AA and EPA, respectively.

Identification of cases

From the Danish National Patient Registry and/or the Danish Causes of Death Registry we identified all participants in the cohort who were registered with a first time diagnosis of MI, according to the International Classification of Disease (ICD) 8 (410.00-410.99) or ICD-10 (I21.0-

I21.9) coding, during the study period. Furthermore, all cases of cardiac arrest (ICD-8: 427.27 or ICD-10: I46.0-I46.9) were included if the arrest was considered to be of cardiac origin after validation in each individual case. An earlier study from our department validated the MI diagnosis from baseline through 2003 by complete review of all medical records and found a positive predictive value above 92% when the diagnoses were obtained from a hospital ward [26]. All validated cases of MI from the validation study were readily accepted as cases for the present study. From January 2004 through July 2013, all participants with an incident MI diagnosed from a ward were accepted as cases without further validation. All other diagnoses of incident MI and cardiac arrest were validated by reviewing a complete list of diagnoses and interventional procedures recorded in the Danish National Patient Registry for each potential case.

Statistics

Regarding the *ALOX-5* tandem repeat polymorphism we considered five repeats or more (5-6 repeats) as the wild type allele and alleles with less than five repeats (2-4 repeats) as variant alleles. We planned a priory to analyse each copy-number variant separately and collectively according to the above definition of variant and wild type alleles.

Polymorphisms were categorised according to genotype and analysed with one degree of freedom, assuming a recessive model of inheritance. Allele frequencies were tested for Hardy–Weinberg equilibrium (HWE) in the sub-cohort using a chi-square test (X^2-test).

Measures of association were assessed using Cox proportional hazards multivariate regression models with age as the time axis and delayed entry. In accordance with the case-cohort design, we used a weighting scheme and robust variance estimates as described by Kalbfleisch and Lawless [27]. Analyses were conducted for the whole cohort and also separately for men and women. Participants were treated as at risk from baseline until either MI, death, emigration or end of follow-up occurred.

To investigate potential interaction between genotype and adipose tissue content of EPA and AA, we cross-tabulated genotype by quintiles of EPA or AA content. Interaction was assessed quantitatively by calculation of the "relative excess risk due to interaction" (RERI) that describes additive interaction under the sufficient cause model [28,29]. We calculated robust variance estimates using bootstrap with 1000 replications for each model.

To address potential confounding, we adjusted for traditional risk factors for MI (model A2) including smoking habits (never, former or current (<15 g/day, 15-25 g/day, >25 g/day) smoker), body mass index (kg/m^2), waist circumference (cm), physical activity (hours/week), alcohol intake (g/day), educational level (basic school, higher education: 1-3 years or >3 years) and, for women, menopausal status (pre- or post-menopausal). Additionally, we adjusted for medical

history (model B) or dietary variables (model C) in separate models. Regarding medical history, we included history of diabetes mellitus (yes/no), hypertension (yes/no) and hypercholesterolaemia (yes/no) complemented with use of anti-diabetic and/or lipid-lowering drugs. Additional dietary variables included dietary fiber (g/day), adipose tissue content of total saturated, monounsaturated, and trans fatty acids (percentages of total fatty acids). All continuous variables were included in the models as restricted cubic splines with five knots. Potential confounders were selected a priori based on current knowledge of the 5-LOX pathway and risk factors for MI.

The proportional hazards assumption was checked by visual inspection of log-log plots and by evaluation of scaled Schoenfeld residuals versus time. P-values (two-tailed) < 0.05 were considered statistically significant. STATA, version 14.1 (StataCorp, College Station, TX, US) was used as statistical software.

5. Results

Population characteristics

In total, 57,053 (35%) subjects accepted the invitation and were enrolled into the study. Initially 1,506 were excluded because of missing baseline questionnaires (*n*=42), if participants were identified with a cancer diagnosis in the Danish National Patient Registry before baseline (*n*=564) or if registered with MI or cardiac arrest before inclusion (*n*=900). We identified 3,089 cases of incident MI during a median follow-up time of 17.0 years. Subjects for whom information regarding one or more covariates used in the adjusted analyses were missing (*n*=346), missing DNA-samples (*n*=255) and subjects with missing adipose tissue data (*n*=376) were excluded. In total, 2,680 cases were included in the analyses, but not all samples were successfully genotyped, and therefore, 103 and 86 samples were missing for rs12762303 and rs59439148, respectively.

Table 1 describes the cases and the sub-cohort with respect to important baseline characteristics. Cases had a higher median age, BMI, waist circumference and a higher proportion of smokers, while weekly physical activity and educational level were lower compared to the sub-cohort. More cases suffered from hypertension, hypercholesterolemia and diabetes mellitus (Table 1).

Association of *ALOX-5* tandem repeat polymorphism with MI

We genotyped the tandem repeat rs59439148 and calculated genotype frequencies, according to the number of hexamer-repeats ('-CCCGCC-') for the two alleles (Table 2). The 5-repeats allele was, by far, the most common allele (84.4%) and considered as the wild type. Next, the 4-repeats allele was the most frequent variant observed (15.2%), whereas alleles with less than 4-repeats were rare (<1%). As a consequence of the observed allele frequencies, we analysed the tandem repeat

defining the variant allele as alleles with less than 5 repeats (2-4 repeats) and the wild type as alleles with 5 repeats or more (5-6 repeats). Table 3 summarises the allele frequencies of the polymorphism according to our definition of variant/wild type.

Table 4 shows the cross-tabulation of genotype by quintiles (Q) of EPA or AA content in adipose tissue. The table displays hazard ratios for the association with incident MI. Looking at AA, we found a positive association with MI when comparing Q1 with Q5 for both the reference genotype and homozygotes of the variant. Furthermore, the variant genotype was associated with a higher risk of MI, comparing homozygotes of the variant with carriers of the wild type. This association seemed to be augmented with higher content of AA, in particular for the highest quintile (Q5) of AA content where we found a threefold higher risk of MI among homozygotes for the variant allele compared with the reference (HR=3.02 95% CI: 1.41;6.44 (model B)). Concerning EPA, we observed a trend towards a negative association with MI for increasing content of EPA. Accordingly, we selected the highest quintile (Q5) as reference in table 4 to better interpret the interaction and found low EPA contents to be associated with a higher risk of MI (HR=1.29, 95%CI: 1.05;1,58) comparing Q1 with the reference (model B). For the reference group with high EPA content, carrier status for the polymorphism did not affect the risk of MI, but for lower quintiles of EPA carrier status was positively associated with MI for homozygous carriers of the variant compared with carriers of the wild type, most pronounced for Q1 compared to the reference with a HR of 2.15 (95% CI: 0.91;5.09) in model B. Associations differed slightly between models.

We also performed sex-stratified analyses and found some discrepancies between men and women (Supplementary, Table 4a+b). For women, we observed substantial variations between models with no evident trend across quintiles and large CIs.

Additive measures of interaction between EPA or AA and *ALOX-5* promoter polymorphism

To evaluate a possible interaction between EPA or AA and genotype quantitatively, we calculated the RERI (Table 5). These values reflected the measures of association from Table 4, but estimation of robust variance measures by bootstrap revealed no significant interactions between the content of EPA or AA and genotype.

Linkage between ALOX-5 tandem repeat polymorphism and rs12762303

We analysed the association between the SNP, rs12762303, and incident MI, finding very similar measures of association to those of the tandem repeat polymorphism (Supplementary, Table 6). Using the Haploview software [31], we found this SNP to be in almost perfect LD with the tandem repeat polymorphism (*D'*=0.99).

6. Discussion

Based on the Danish, Diet, Cancer and Health Cohort, we conducted a case-cohort study to investigate the interaction between adipose tissue content of EPA and AA and the *ALOX-5* tandem repeat polymorphism on incident MI. Cross-tabulation of genotype by quintiles of EPA or AA content indicated a higher risk of MI for carriers of two variant alleles. While the association between genotype and MI seemed to be augmented by higher content of AA compared with the reference group with a low content of AA, the opposite was observed for EPA where the association seemed to be attenuated by increasing content of EPA. However, interaction on an additive scale assessed by calculation of RERIs was not nominally significant.

Strengths and limitations

The Diet, Cancer and Health study is a large prospective cohort study, and while earlier studies on the 5-LOX pathway used a retrospective study design, this study holds the advantages of the prospective design. This might be of importance, in particular when assessing combined effects of genetics and diet because dietary factors are more sensitive to confounding than genotype information alone. Exposure to EPA and AA was assessed by adipose tissue content of the respective fatty acids which reflects the average long-term (1-3 years) exposure to these fatty acids, and as a biomarker it may represent the true endogenous exposure more precisely than a dietary assessment of fatty acids intake [13,14]. Concerning outcome assessment, there was a very limited loss to follow-up, and all diagnoses of MI or cardiac arrest were either validated from review of medical records or from a complete list of diagnoses and medical procedure codes, ensuring a high validity of cases. The cohort was well characterised at baseline regarding demographic data, anthropometrics, dietary and other lifestyle factors, which allowed for adjustment of potential confounding [21].

We applied adjustments for potential confounding in different layers. First, a basic layer adjusting for well-established demographic and lifestyle risk factors for MI (model A2). Next, we applied an additional layer including medical history (model B) or dietary factors (model C). Only minor variations between models were observed, and generally, measures of association and the conclusions were similar for the various models. We consider model B as our primary model, but all models are included in the tables.

The study also had limitations. Even though the study included a high number of cases with both genotype and adipose tissue information, the number of homozygote carriers of the variant genotype was limited, in particular when cross-tabulated by quintiles of fatty acids content. This made assessment of interaction difficult, and stratifying further by sex did not yield a meaningful comparison in women because of the low number of cases. The median follow-up was

17.0 years, and lifestyle or dietary measures were not re-evaluated during the study period. A long follow-up allowed us to accumulate more outcome events, but subjects might change their lifestyle and habits over time. Also, public awareness of disease prevention and changes in standard medical care might influence on the participants' risk profile and limit our ability to address confounding.

General discussion

Since the Hallmark study by Dwyer et al. from 2004 [15] reported the *ALOX-5* tandem repeat polymorphism (rs59439148) to be associated with higher intima-media thickness, a number of studies have investigated this polymorphism on other relevant endpoints, e.g. patients with MI and coronary artery disease verified by angiography. Notably, two independent case-control studies in MI patients with Northern European origin (Caucasians), did not find an association between MI and variants of the polymorphism [17,18]. In a mixed population of Caucasians and African Americans, Hartiala et. al demonstrated a positive association, but only in African Americans [19]. In general, the previous studies had a limited size and none of the studies examined the influence of diet. A recent study by our group (data not published) investigated the overall association between rs59439148 genotypes and MI and found a positive association when comparing homozygotes for the wildtype with homozygotes for the variant. However, results were only statistically significant in men (HR=1.63, 95% CI: 1.06;2.52), while the pooled analyses were borderline significant (HR=1.35, 95% CI: 0.96;1.90).

In the present study, our results indicated a positive association between carriers of two variant alleles and MI when compared with the reference genotype, but the association was only statistically significant when comparing homozygotes for the variant genotype in the high stratum of AA content with the reference group. Furthermore, the associations were highly dependent on the content of EPA and AA in adipose tissue, where a high content of EPA seemed to blunt the effect of the polymorphism, while a high content of AA seemed to exacerbate the effect. However, measures of interaction on an additive scale were not statistically significant. The initial study by Dwyer et. al [15] was also the first study to suggest interaction between dietary intake of 5-LOX substrates and the tandem repeat polymorphism, well in line with our findings. Later, the same group performed a large study on Costa Rican nationals, including 1,885 cases of MI [16], with no overall effect from the variant genotype, but the group with a high intake of AA had a significantly higher risk of MI when the variant genotype was present indicating interaction between AA intake and genotype. No interaction could be detected for marine n-3 PUFA. Both studies used dietary questionnaires to assess the intake of fatty acids, while our study used adipose tissue content as a biomarker reflecting long-term dietary intake or, more precisely, the endogenous exposure to the respective fatty acids.

In addition to the tandem repeat polymorphism, we genotyped the SNP rs12762303 that is located only 409 base pairs upstream from the tandem repeat and was previously described to be in close LD with rs59439148 [30], confirming that these two polymorphisms were in almost perfect LD. In future studies, it may be relevant to genotype the more cost-efficient SNP, rs12762303, instead of the tandem repeat polymorphism.

Studies have examined other polymorphisms in ALOX-5 [19,30], but none of these, other than rs59439148 and SNPs linked to this polymorphism, has been associated with atherosclerosis traits. Furthermore, a number of studies have supported a role for other candidate genes in the 5-LOX pathway [32–34], but replication studies have been inconclusive, and despite great efforts, studies have not been able to agree upon functional polymorphisms in any of the other candidate genes, except *ALOX-5*.

One of the key mechanisms linking the 5-LOX pathway to the development of atherosclerosis may be attributed to the formation of LTs and their pro-inflammatory properties. LTs increase vascular permeability and act as potent chemo attractants among other pro-inflammatory functions [4,35]. Furthermore, a high expression of multiple 5-LOX enzymes has been found in human atherosclerotic plaques and linked to the stage of plaque development [36,37]. Genetic association studies have also provided some evidence of a link between the 5-LOX pathway and atherosclerotic disease, and in this regard, our study adds to the body of evidence in support of this hypothesis, confirming the tandem repeat polymorphism in *ALOX-5* (rs59439148) as a functional polymorphism. Finally, our results indicated an interaction between fatty acid substrates for the 5-LOX enzymes and rs59439148 which is well in line with the hypothesis that the differential roles of marine n-3 PUFA and AA in atherosclerotic disease states may, at least partly, be explained by the formation of less inflammatory LTs derived from EPA compared to AA. The present study also adds mechanistic evidence for the role of marine n-3 PUFA and AA in MI through the 5-LOX system.

Conclusion

The present study indicated that homozygote carriers of the variant genotype had a higher risk of incident MI compared with carriers of the wild type, and this association was modulated by adipose tissue content of EPA and AA. Thus, results suggested that the association was augmented by intake of AA, while EPA attenuated the association. However, the interaction was not statistically significant when evaluated by calculation of RERIs.

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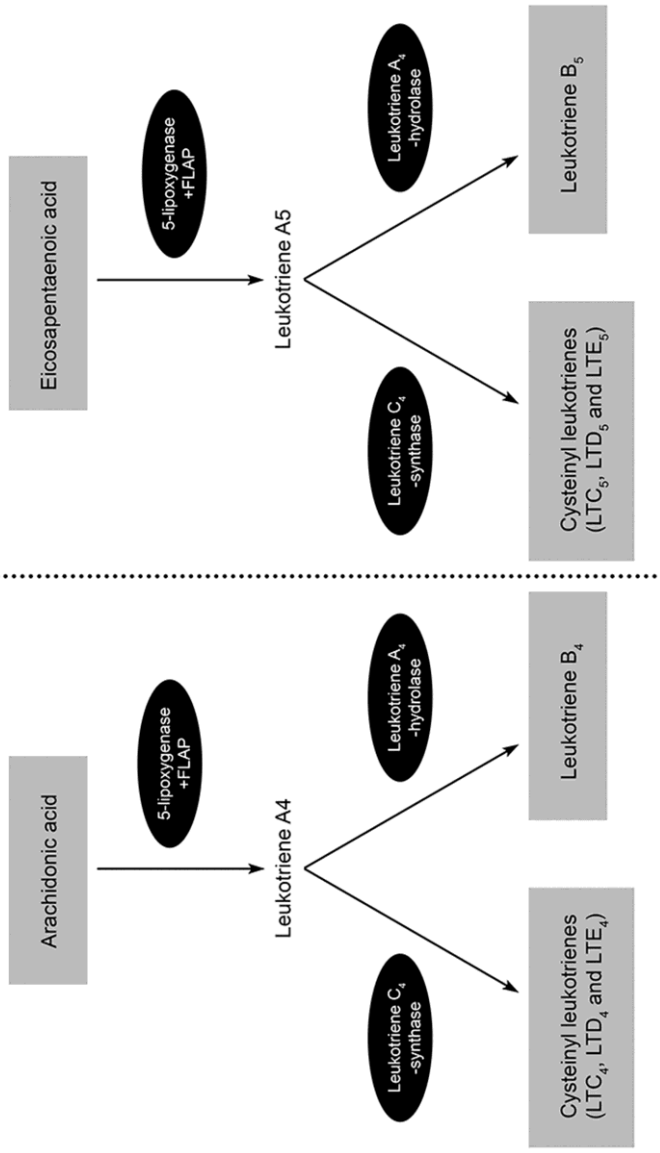

Figure 1. Outline of the 5-lipoxygenase pathway and the production of 4- and 5-series leukotrienes

TABLE 1

Baseline characteristics of the sub-cohort and cases

	Men		Women	
Variable	Sub-cohort (*n*=1,454)	Cases (*n*=1,924)	Sub-cohort (*n*=1,250)	Cases (*n*=756)
Age (years)	56.3 (51.2;63.2)	57.7 (51.7;63.9)	56.3 (51.1;62.9)	59.2 (52.4;64.1)
Physical activity (hours/week)	2.5 (0.0;8.5)	2.0 (0.0;8.0)	2.5 (0.0;8.0)	2.0 (0.0;7.0)
BMI (kg/m2)	26.4 (23.0;31.2)	27.0 (23.4;32.3)	24.6 (20.9;31.1)	26.1 (21.1;33.5)
Waist circumference (cm)	95.5 (86.0;109.0)	97.0 (87.0;112.0)	80.0 (69.0;97.0)	84.0 (70.0;102.0)
Alcohol intake (g/day)	19.4 (3.3;61.9)	18.2 (2.5;62.3)	9.4 (1.2;34.8)	6.5 (0.5;32.3)
Smoking (% (n))				
Never smoker	25.8 (375)	18.4 (353)	43.7 (546)	28.3 (214)
Former smoker	35.4 (515)	29.5 (567)	22.8 (285)	19.3 (146)
<15 g/day	11.4 (165)	12.9 (248)	16.1 (201)	22.1 (167)
15-25 g/day	16.6 (242)	23.5 (453)	14.7 (184)	25.0 (189)
>25 g/day	10.8 (157)	15.8 (303)	2.7 (34)	5.3 (40)
Educational level (% (n))				
Basic school	33.9 (493)	43.4 (835)	32.0 (400)	44.2 (334)
Higher education, 1-3 years	42.4 (617)	37.1 (713)	49.7 (621)	46.4 (351)
Higher education, >3 years	23.7 (344)	19.5 (376)	18.3 (229)	9.4 (71)
Menopausal status (% (n))				
Post-menopausal	- -	- -	59.4 (742)	70.1 (530)
Pre-menopausal	- -	- -	31.4 (392)	17.1 (129)
Medical history (% (n))				
Hypertension	14.8 (215)	22.6 (434)	16.7 (209)	31.8 (240)
Hypercholesterolaemia	8.5 (123)	12.3 (236)	6.2 (77)	13.4 (101)
Diabetes mellitus	3.0 (43)	5.2 (99)	1.4 (17)	4.5 (34)
Adipose tissue (%, of total fatty acids)				
EPA	0.1 (0.1;0.2)	0.1 (0.1;0.2)	0.1 (0.1;0.2)	0.1 (0.1;0.2)
AA	0.3 (0.2;0.5)	0.4 (0.2;0.5)	0.4 (0.3;0.5)	0.4 (0.3;0.6)

Abbreviations: BMI, Body mass index; EPA, Eicosapentaenoic acid; AA, Arachidonic acid.

Continuous variables are reported as medians (10th;90th percentile) and categorical variables as percent (n).

TABLE 2

Distribution of genotypes for rs59439148, according to number of tandem repeats (5'-GGGCGG-3')

Genotype	Sub cohort	Cases
22	- -	- -
23	- -	- -
24	2 (0.08)	2 (0.08)
25	16 (0.60)	10 (0.38)
26	- -	- -
33	- -	- -
34	3 (0.11)	1 (0.04)
35	1 (0.04)	3 (0.11)
36	- -	- -
44	71 (2.66)	78 (2.97)
45	671 (25.17)	630 (23.98)
46	- -	- -
55	1,902 (71.34)	1,902 (72.40)
56	- -	- -
66	- -	1 (0.04)
Total	2,666	2,627

Reported as number of subjects with frequencies in parentheses (%). No observations are indicated with a dash.

TABLE 3

Distribution of alleles for rs59439148 and rs12762303, *ALOX-5* promoter polymorphisms

			Men		Women		All	
Polymorphism	Genomic position	Allele	Sub cohort	Cases	Sub cohort	Cases	Sub cohort	Cases
rs59439148	10: 4537413(2-7)	V/W	15.4 (432)	15.6 (589)	16.6 (408)	14.5 (216)	15.8 (840)	15.3 (805)
rs12762303	10: 45373723	C/T	14.1 (402)	14.6 (549)	15.8 (388)	13.9 (205)	14.9 (790)	14.4 (754)

Abbreviations: *ALOX-5*, arachidonate 5-lipoxygenase; V, variant allele; W, wild type allele.

Results presented as allele frequencies (n) for the minor allele (underlined). Rs59439148 alleles were defined as wildtype if the number of hexamere-repeats (5'-GGGCGG-3') were five or larger (5-6 repeats) and as variant if less than five repeats (2-4 repeats). Alleles correspond to the positive DNA-strand and genomic position are obtained from dbSNP, human assembly GRCh38.p2.

TABLE 4

Cross-tabulation of adipose tissue content of EPA or AA and genotype of tandem-repeat rs59439148, showing HRs for the association with MI

	Genotype							
	Model A1*		Model A2**		Model B***		Model C****	
EPA	W/W, W/V	V/V	W/W, W/V	V/V	W/W, W/V	V/V	W/W, W/V	V/V
Q5	1 (ref)	0.85 (0.39;1.82)	1 (ref)	0.89 (0.39;2.02)	1 (ref)	1.16 (0.52;2.59)	1 (ref)	0.85 (0.37;1.96)
Q4	1.18 (0.98;1.41)	1.02 (0.50;2.06)	1.12 (0.92;1.35)	1.20 (0.60;2.41)	1.17 (0.96;1.43)	1.31 (0.63;2.74)	1.17 (0.96;1.43)	1.32 (0.66;2.65)
Q3	1.09 (0.90;1.32)	1.50 (0.53;4.26)	0.95 (0.77;1.17)	1.59 (0.61;4.13)	1.03 (0.83;1.28)	1.62 (0.59;4.46)	1.04 (0.83;1.29)	1.61 (0.61;4.23)
Q2	1.26 (1.06;1.51)	1.88 (1.00;3.53)	1.12 (0.92;1.36)	1.83 (0.98;3.43)	1.24 (1.01;1.52)	1.82 (0.90;3.65)	1.23 (1.00;1.51)	2.03 (1.08;3.84)
Q1	1.30 (1.09;1.55)	2.31 (1.08;4.96)	1.18 (0.97;1.43)	1.70 (0.69;4.15)	1.29 (1.05;1.58)	2.15 (0.91;5.09)	1.31 (1.06;1.62)	1.92 (0.76:4.83)
AA								
Q1	1 (ref)	1.48 (0.69;3.16)	1 (ref)	1.37 (0.61;3.07)	1 (ref)	1.26 (0.52;3.07)	1 (ref)	1.37 (0.60;3.12)
Q2	1.08 (0.90;1.29)	1.30 (0.65;2.60)	1.07 (0.88;1.29)	1.29 (0.60;2.79)	1.05 (0.87;1.27)	1.37 (0.66;2.86)	1.09 (0.89;1.32)	1.31 (0.61;2.86)
Q3	1.26 (1.05;1.50)	1.18 (0.56;2.49)	1.18 (0.97;1.43)	1.15 (0.55;2.39)	1.16 (0.95;1.42)	1.15 (0.55;2.42)	1.20 (0.98;1.46)	1.11 (0.53;2.35)
Q4	1.46 (1.21;1.75)	1.39 (0.64;3.02)	1.24 (1.01;1.52)	1.60 (0.71;3.60)	1.16 (0.93;1.43)	1.50 (0.65;3.50)	1.24 (1.00;1.53)	1.66 (0.74;3.72)
Q5	1.61 (1.35;1.93)	2.91 (1.40;6.03)	1.34 (1.09;1.66)	2.57 (1.19;5.54)	1.17 (0.94;1.46)	3.02 (1.41;6.44)	1.32 (1.05;1.65)	2.50 (1.14;5.52)

Abbreviations: HR, hazard ratio; EPA, eicospentaenoic acid; AA, arachidonic acid; W, wildtype allele; V, variant allele.

Hazardratioes with 95 % conficende intervals in (parentheses) from Cox proportional hazards model cross tabulated by quintiles of EPA or AA in adipse tissue and genotype of rs59439148. The reference group was the lowest quintile of fattyacids and carriers of one or two wildtype alleles.

*Model A1: Crude analyses adjusted for sex.

**Model A2: Adjusted for lifestyle and demographic measures, including sex, smoking status, educational level, physical activity, BMI, waist circumferrence and alcohol consumption.

***Model B: Adjusted as model A2 with additional medical history variables, including History of diabetes mellitus, hypertention and hypercholesterolaemia.

****Model C: Adjusted as model A2 with additional dietary variables, including adipose tissue content of total saturated, monunsaturated and trans fatty acids and dietary fiber.

TABLE 5

Interaction between rs59439148 and content of EPA/AA, evaluated on an additive scale using relative excess risk due to interaction (RERI)

	Genotype							
	Model A1*		Model A2**		Model B***		Model C****	
EPA	W/W, W/V	V/V	W/W, W/V	V/V	W/W, W/V	V/V	W/W, W/V	V/V
Q5	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Q4	n/a	-0.01 (-1.20;1.18)	n/a	0.19 (-1.10;1.49)	n/a	-0.02 (-1.65;1.62)	n/a	0.29 (-1.18;1.76)
Q3	n/a	0.57 (-2.33;3.47)	n/a	0.75 (-1.56;3.06)	n/a	0.44 (-2.93;3.81)	n/a	0.72 (-1.92;3.37)
Q2	n/a	0.77 (-0.78;2.32)	n/a	0.81 (-0.71;2.34)	n/a	0.42 (-1.49;2.34)	n/a	0.95 (-0.81;2.72)
Q1	n/a	1.17 (-1.15;3.49)	n/a	0.63 (-2.00;3.26)	n/a	0.71 (-2.29;3.71)	n/a	0.76 (-2.06;3.58)
AA								
Q1	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Q2	n/a	-0.26 (-2.04;1.52)	n/a	-0.14 (-2.09;1.81)	n/a	0.06 (-1.93;2.06)	n/a	-0.15 (-2.37;2.08)
Q3	n/a	-0.56 (-2.31;1.19)	n/a	-0.40 (-2.29;1.50)	n/a	-0.27 (-2.13;1.59)	n/a	-0.46 (-2.52;1.60)
Q4	n/a	-0.55 (-2.50;1.41)	n/a	-0.00 (-2.26;2.25)	n/a	0.09 (-2.19;2.36)	n/a	0.05 (-2.63;2.73)
Q5	n/a	0.81 (-2.52;4.15)	n/a	0.86 (-2.12;3.84)	n/a	1.59 (-1.62;4.80)	n/a	0.81 (-2.32;3.95)

Abbreviations: RERI, relative excess risk due to interaction; EPA, eicospentaenoic acid; AA, arachidonic acid; W, wildtype allele; V, variant allele.
RERI with 95 % conficende intervals in (parentheses) from Cox proportional hazards model cross tabulated by quintiles of EPA or AA in adipse tissue and genotype of rs59439148.

*Model A1: Crude analyses adjusted for sex.

**Model A2: Adjusted for lifestyle and demographic measures, including sex, smoking status, educational level, physical activity, BMI, waist circumferrence and alcohol consumption.

***Model B: Adjusted as model A2 with additional medical history variables, including History of diabetes mellitus, hypertention and

****Model C: Adjusted as model A2 with additional dietary variables, including adipose tissue content of total saturated, monunsaturated and trans fatty acids and dietary fiber.

SUPPLEMENTARY, TABLE 4A (men)

Cross-tabulation of adipose tissue content of EPA or AA and genotype of tandem-repeat rs59439148, showing HRs for the association with MI

	Genotype							
	Model A1*		Model A2**		Model B***		Model C****	
EPA	W/W, W/V	V/V	W/W, W/V	V/V	W/W, W/V	V/V	W/W, W/V	V/V
Q5	1 (ref)	0.86 (0.31;2.40)	1 (ref)	0.81 (0.27;2.37)	1 (ref)	1.06 (0.37;3.09)	1 (ref)	0.80 (0.27;2.39)
Q4	1.11 (0.89;1.40)	1.10 (0.42;2.88)	1.05 (0.83;1.34)	1.45 (0.55;3.78)	1.04 (0.81;1.33)	1.45 (0.53;4.01)	1.11 (0.86;1.42)	1.63 (0.63;4.19)
Q3	1.01 (0.80;1.26)	1.10 (0.44;2.78)	0.90 (0.71;1.15)	1.33 (0.54;3.28)	0.98 (0.77;1.26)	1.35 (0.53;3.40)	0.99 (0.77;1.28)	1.43 (0.57;3.59)
Q2	1.22 (0.99;1.52)	3.01 (1.15;7.87)	1.11 (0.88;1.40)	3.10 (1.24;7.75)	1.18 (0.93;1.50)	2.48 (0.85;7.30)	1.22 (0.95;1.56)	3.62 (1.45;9.02)
Q1	1.23 (0.99;1.53)	2.51 (0.89;7.09)	1.15 (0.91;1.47)	1.97 (0.61;6.32)	1.22 (0.95;1.57)	2.41 (0.78;7.46)	1.28 (0.98;1.68)	2.33 (0.69;7.83)
AA								
Q1	1 (ref)	2.38 (0.85;6.63)	1 (ref)	2.21 (0.71;6.82)	1 (ref)	1.67 (0.44;6.26)	1 (ref)	2.42 (0.76;7.70)
Q2	1.07 (0.86;1.32)	1.41 (0.57;3.45)	1.06 (0.85;1.33)	1.54 (0.58;4.05)	1.04 (0.83;1.31)	1.69 (0.67;4.27)	1.08 (0.86;1.37)	1.61 (0.60;4.27)
Q3	1.21 (0.97;1.51)	1.34 (0.53;3.40)	1.15 (0.91;1.47)	1.26 (0.50;3.13)	1.15 (0.90;1.47)	1.29 (0.51;3.24)	1.20 (0.93;1.53)	1.31 (0.51;3.33)
Q4	1.34 (1.08;1.68)	0.81 (0.30;2.22)	1.11 (0.87;1.42)	0.94 (0.33;2.66)	1.04 (0.80;1.33)	0.86 (0.29;2.51)	1.12 (0.87;1.45)	1.00 (0.35;2.82)
Q5	1.53 (1.22;1.91)	4.09 (1.54;10.84)	1.30 (1.00;1.69)	4.04 (1.45;11.29)	1.13 (0.86;1.49)	4.47 (1.67;11.95)	1.30 (0.98;1.71)	3.81 (1.32;10.95)

SUPPLEMENTARY, TABLE 4B (women)

Cross-tabulation of adipose tissue content of EPA or AA and genotype of tandem-repeat rs59439148, showing HRs for the association with MI

	Genotype							
	Model A1*		Model A2**		Model B***		Model C****	
EPA	W/W, W/V	V/V	W/W, W/V	V/V	W/W, W/V	V/V	W/W, W/V	V/V
Q5	1 (ref)	0.87 (0.27;2.87)	1 (ref)	1.18 (0.35;3.96)	1 (ref)	1.41 (0.40;4.95)	1 (ref)	1.22 (0.37;4.07)
Q4	1.49 (1.10;2.03)	1.46 (0.40;5.25)	1.35 (0.96;1.90)	1.00 (0.25;4.08)	1.58 (1.10;2.27)	1.41 (0.35;5.61)	1.40 (0.99;2.00)	1.13 (0.26;4.81)
Q3	1.43 (1.05;1.95)	2.92 (0.72;11.93)	1.25 (0.88;1.76)	2.46 (0.51;11.87)	1.36 (0.95;1.97)	2.20 (0.38;12.70)	1.40 (0.97;2.02)	2.51 (0.50;12.68)
Q2	1.48 (1.08;2.02)	0.78 (0.27;2.31)	1.17 (0.82;1.66)	0.58 (0.18;1.86)	1.31 (0.90;1.90)	0.73 (0.23;2.33)	1.29 (0.89;1.87)	0.64 (0.19;2.18)
Q1	1.45 (1.06;1.98)	1.91 (0.51;7.18)	1.20 (0.84;1.71)	2.24 (0.61;8.18)	1.42 (0.97;2.07)	2.84 (0.77;10.48)	1.32 (0.89;1.95)	2.87 (0.78;10.60)
AA								
Q1	1 (ref)	0.46 (0.09;2.23)	1 (ref)	0.40 (0.07;2.25)	1 (ref)	0.46 (0.08;2.51)	1 (ref)	0.41 (0.07;2.46)
Q2	1.25 (0.92;1.70)	0.83 (0.28;2.45)	1.35 (0.97;1.90)	0.95 (0.30;3.01)	1.28 (0.90;1.81)	1.00 (0.32;3.15)	1.41 (0.99;2.01)	1.00 (0.31;3.28)
Q3	1.26 (0.93;1.70)	2.21 (0.72;6.77)	1.36 (0.97;1.90)	1.48 (0.41;5.26)	1.30 (0.92;1.84)	1.21 (0.33;4.44)	1.41 (0.99;2.01)	1.40 (0.38;5.23)
Q4	1.44 (1.06;1.96)	0.88 (0.26;2.94)	1.35 (0.94;1.94)	1.05 (0.30;3.72)	1.27 (0.88;1.84)	1.16 (0.33;4.07)	1.43 (0.98;2.10)	1.23 (0.34;4.39)
Q5	1.77 (1.31;2.38)	7.07 (1.77;28.26)	1.49 (1.03;2.16)	7.64 (1.63;35;76)	1.28 (0.87;1.88)	8.71 (1.85;40.99)	1.53 (1.04;2.25)	9.48 (2.10;42.77)

Abbreviations: EPA, eicospentaenoic acid; AA, arachidonic acid; W, wildtype allele; V, variant allele.

Hazardratioes with 95 % conficende intervals in (parentheses) from Cox proportional hazards model cross tabulated by quintiles of EPA or AA in adipse tissue and genotype of rs59439148. The reference group was the lowest quintile of fattyacids and carriers of one or two wildtype alleles.

*Model A1: Crude analyses adjusted for sex.

**Model A2: Adjusted for lifestyle and demographic measures, including smoking status, educational level, physical activity, BMI, waist circumferrence and alcohol consumption.

***Model B: Adjusted as model A2 with additional medical history variables, including History of diabetes mellitus, hypertention and hypercholesterolaemia.

****Model C: Adjusted as model A2 with additional dietary variables, including adipose tissue content of total saturated, monunsaturated and trans fatty acids and dietary fiber.

SUPPLEMENTARY, TABLE 6

Cross-tabulation of adipose tissue content of EPA or AA and genotype of rs12762303, showing HRs for the association with MI

	Genotype							
	Model A1*		Model A2**		Model B***		Model C****	
EPA	W/W, W/V	V/V	W/W, W/V	V/V	W/W, W/V	V/V	W/W, W/V	V/V
Q5	1 (ref)	0.90 (0.41;1.97)	1 (ref)	0.98 (0.43;2.24)	1 (ref)	1.22 (0.54;2.76)	1 (ref)	0.95 (0.41;2.19)
Q4	1.18 (0.98;1.41)	0.97 (0.41;2.27)	1.12 (0.93;1.36)	1.23 (0.53;2.85)	1.18 (0.97;1.44)	1.25 (0.51;3.10)	1.18 (0.97;1.43)	1.34 (0.58;3.13)
Q3	1.09 (0.90;1.33)	1.40 (0.42;4.63)	0.95 (0.77;1.18)	1.47 (0.49;4.43)	1.03 (0.83;1.29)	1.41 (0.45;4.42)	1.04 (0.84;1.30)	1.55 (0.50;4.78)
Q2	1.27 (1.06;1.51)	2.63 (1.12;6.22)	1.13 (0.93;1.37)	2.09 (0.92;4.74)	1.24 (1.01;1.51)	2.52 (1.09;5.83)	1.24 (1.01;1.52)	2.35 (1.05;5.29)
Q1	1.30 (1.09;1.55)	2.48 (1.03;6.02)	1.18 (0.97;1.43)	1.82 (0.63;5.32)	1.29 (1.05;1.58)	2.52 (0.90;7.09)	1.31 (1.06;1.62)	2.11 (0.69;6.54)
AA								
Q1	1 (ref)	2.37 (0.87;6.45)	1 (ref)	1.72 (0.56;5.33)	1 (ref)	2.05 (0.70;6.06)	1 (ref)	1.79 (0.57;5.67)
Q2	1.08 (0.90;1.29)	1.46 (0.68;3.15)	1.06 (0.88;1.29)	1.50 (0.63;3.57)	1.05 (0.87;1.27)	1.61 (0.71;3.64)	1.08 (0.89;1.31)	1.54 (0.64;3.75)
Q3	1.26 (1.05;1.50)	1.28 (0.55;2.93)	1.18 (0.97;1.43)	1.31 (0.59;2.91)	1.16 (0.95;1.42)	1.27 (0.56;2.89)	1.19 (0.97;1.45)	1.31 (0.59;2.91)
Q4	1.46 (1.22;1.76)	1.13 (0.42;3.02)	1.25 (1.02;1.53)	1.29 (0.45;3.64)	1.17 (0.94;1.44)	1.20 (0.40;3.59)	1.24 (1.01;1.54)	1.33 (0.47;3.73)
Q5	1.63 (1.36;1.95)	2.35 (1.05;5.22)	1.36 (1.10;1.68)	2.07 (0.89;4.81)	1.18 (0.95;1.48)	2.45 (1.07;5.64)	1.33 (1.06;1.66)	2.00 (0.84;4.78)

Abbreviations: EPA, eicospentaenoic acid; AA, arachidonic acid; W, wildtype allele; V, variant allele.

Hazardratioes with 95 % conficende intervals in (parentheses) from Cox proportional hazards model cross tabulated by quintiles of EPA or AA in adipse tissue and genotype of rs12762303. The reference group was the lowest quintile of fattyacids and carriers of one or two wildtype alleles.

*Model A1: Crude analyses adjusted for sex.

**Model A2: Adjusted for lifestyle and demographic measures, including sex, smoking status, educational level, physical activity, BMI, waist circumferrence and alcohol consumption.

***Model B: Adjusted as model A2 with additional medical history variables, including History of diabetes mellitus, hypertention and hypercholesterolaemia.

****Model C: Adjusted as model A2 with additional dietary variables, including adipose tissue content of total saturated, monunsaturated and trans fatty acids and dietary fiber.

ISSN (online): 2246-1302
ISBN (online): 978-87-7112-785-0

AALBORG UNIVERSITY PRESS