Bioactive fatty acids and coronary heart disease

Mechanisms and clinical effects of dietary fatty acids

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Scientific environment

The studies presented in this thesis are based on the collaboration between Section for Cardiology under Professor Ottar Nygård and Section for Medical Biochemistry under Professor Rolf Kristian Berge. Both sections belong to the Department of Clinical Science at the University of Bergen.

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Abstract

Background: A high intake of omega-3 (n-3) long-chain polyunsaturated fatty acids (LCPUFAs), which are potential peroxisome proliferator-activated receptor (PPAR) agonists, has been associated with proposed favourable effects related to prevention and treatment of coronary heart disease. The n-3 LCPUFAs eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids are poorly oxidizable and resemble the effects of the modified fatty acid and pan-PPAR agonist tetradecylthioacetic acid (TTA) mainly through PPAR activation.

Aims: Aims were to investigate the dietary intake of n-3 LCPUFAs and risk of future coronary events in patients with coronary artery disease (CAD) and also to try and elucidate the mechanistic effects of PPAR activation using a rodent model.

Subjects and Methods: The human studies were sub-studies of participants from the Western Norway B-Vitamin Intervention Trial, who completed a food frequency questionnaire at baseline, from which daily intake of n-3 LCPUFAs [EPA, docosapentaenoic acid (DPA), and DHA] was estimated based on diet and supplements. A variety of blood markers were also measured. The association between intake of n-3 LCPUFAs and subsequent risk of coronary events was investigated in two papers. In **Paper 1** including 2412 patients, the main endpoint was a composite of coronary events. Acute myocardial infarction (AMI) was the outcome in **Paper 2** including 2378 patients, who were sub-grouped as having no diabetes [glycosylated haemoglobin (HbA1c) <5.7%], pre-diabetes (HbA1c ≥5.7%), or diabetes (previous diabetes, fasting baseline serum glucose ≥7.0, or non-fasting glucose ≥11.1 mmol/L). An animal study was used to investigate the long-term effects of the pan-PPAR agonist TTA and/or high-dose fish oil (FO) on cardiac fatty acid (FA) composition and lipid metabolism (**Paper 3**). Male Wistar rats were given different diets containing 25% (w/v) fat: control diet; TTA diet; FO diet; or diet containing both TTA and FO.

Results: Risk of experiencing an endpoint was evaluated by Cox regression over quartiles (**Paper 1**) or tertiles (**Paper 2**). Mean ± SD n-3 LCPUFA intake was 0.58 ± 0.29, 0.83 ± 0.30, 1.36 ± 0.44, and 2.64 ± 1.18 g/day in quartiles 1-4, and 0.43 ± 0.24, 1.08 ± 0.37, and 2.38 ± 1.15 g/day in tertiles 1-3, respectively. There was no overall association between dietary n-3 LCPUFA intake and coronary events in the total human cohort (**Paper 1**). However, a post hoc additive proportional hazards model demonstrated a slightly increased risk of coronary events in participants having an intake of n-3 LCPUFAs <~300 mg/day. Among patients diagnosed with diabetes there was a significantly reduced risk of AMI in those with a high n-3 LCPUFA intake, and there was also a dose-response relation across n-3 LCPUFA tertiles (**Paper 2**). In contrast, among non-diabetic patients with HbA1c <5.7%, a high n-3 LCPUFA intake tended to be associated with an increased risk of AMI, which was significant for fatal AMI and associated with lower HbA1c. The main limitations of the human studies were their observational design and a limited event rate, particularly in the non-diabetic group. In the rat model (**Paper 3**), a long-term diet containing TTA or FO induced an increase in cardiac n-3 LCPUFA composition. Several other cardiac FAs, enzymes, and genes were also changed following TTA and/or FO treatment, indicating increased cardiac FA oxidation.

Conclusions: No risk reduction of coronary events or mortality was observed with high intakes of n-3 LCPUFAs in the total population of patients with CAD. However, a high intake of n-3 LCPUFAs was associated with a reduced risk of AMI in diabetic patients, but with an increased risk of fatal AMI in those without diabetes who had HbA1c <5.7%. Long-term treatment with the pan-PPAR agonist TTA, which has its main effect on PPARα, or high-dose FO, having effects on both PPARα and PPARγ, induced marked changes on cardiac FA metabolism.

Consequences: Further studies should investigate whether patients with diabetes may benefit from having a high intake of n-3 LCPUFAs and whether certain patients with normal glucose tolerance may be careful with a very high intake of these FAs. Because excess PPAR stimulation by FAs other than n-3 LCPUFAs may affect the

cardiac n-3 LCPUFA composition, the underlying mechanisms should be further evaluated.

Abbreviations

ACE	Angiotensin converting enzyme
ACOX	Fatty acyl-CoA oxidase
ACS	Acute coronary syndrome
ALA	α-linolenic acid
AMI	Acute myocardial infarction
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
Apo A-I	Apolipoprotein A-I
Apo A-II	Apolipoprotein A-II
Apo B	Apolipoprotein B
Apo C-III	Apolipoprotein C-III
Apo E	Apolipoprotein E
ARA	Arachidonic acid
ATGL	Adipose triglyceride lipase
CABG	Coronary artery bypass graft surgery
CACT	Carnitine-acylcarnitine translocase
CAD	Coronary artery disease
CHD	Coronary heart disease
CI	Confidence interval
CoA	Coenzyme A
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A2
CPT	Carnitine palmitoyltransferase
CRAT	Carnitine acetyltransferase
CVD	Cardiovascular disease
CYP	Cytochrome P450
DHA	Docosahexaenoic acid

DPA	Docosapentaenoic acid (n-3)
eGFR	Estimated glomerular filtration rate
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinases
FA	Fatty acid
FABP	Fatty acid binding protein
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
FFQ	Food frequency questionnaire
FO	Fish oil
GAM	Generalized additive model
GC	Gas-liquid chromatography
GPAT	Glycerol-3-phosphate acyltransferase
HbA1c	Glycosylated haemoglobin
HDL	High density lipoprotein
HNF-4α	Hepatocyte nuclear factor 4α
HR	Hazard ratio
IDL	Intermediate density lipoprotein
LA	Linoleic acid
LC/MS/MS	Liquid chromatography/tandem mass spectrometry
LCPUFA	Long-chain polyunsaturated fatty acid
LDL	Low density lipoprotein
LOX	Lipoxygenase
LPL	Lipoprotein lipase
LTA_4	Leukotriene A4
LTA_5	Leukotriene A5
LVEF	Left ventricular ejection fraction
LXR	Liver X receptor
MA	Mead acid
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass

	spectrometry
MUFA	Monounsaturated fatty acids
n-3	Omega-3
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PCI	Percutaneous coronary intervention
PGC-1α	Peroxisome proliferator-activated receptor γ co-activator
PGI_2	Prostacyclin
PGI_3	Prostaglandin I3
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator-response element
PUFA	Polyunsaturated fatty acids
qPCR	Real-time polymerase chain reaction
RXR	9-*cis* retinoic acid receptor
SD	Standard deviation
SFA	Saturated fatty acids
SREBP-1c	Sterol regulatory element binding protein 1c
Statins	3-hydroxy-3-methylglutaryl coenzyme A (CoA) reductase inhibitors
TCA	Tricarboxylic acid
TE	Total energy
TG	Triglyceride
TTA	Tetradecylthioacetic acid
TXA_2	Thromboxane A2
TXA_3	Thromboxane A3
UCP	Uncoupling protein
VLDL	Very low density lipoprotein
WENBIT	Western Norway B-Vitamin Intervention Trial

List of publications

Paper 1

Manger MS, Strand E, Ebbing M, Seifert R, Refsum H, Nordrehaug JE, Nilsen DW, Drevon CA, Tell GS, Bleie Ø, Vollset SE, Pedersen ER, Nygård O (2010): Dietary intake of n-3 long-chain polyunsaturated fatty acids and coronary events in Norwegian patients with coronary artery disease. *Am J Clin Nutr*. Jul;92(1):244-51.

Paper 2

Strand E, Pedersen ER, Svingen GFT, Schartum-Hansen H, Rebnord EW, Bjørndal B, Seifert R, Bohov P, Meyer K, Hiltunen JK, Nordrehaug JE, Nilsen DWT, Berge RK, Nygård O (2013): Dietary intake of n-3 long-chain polyunsaturated fatty acids and risk of myocardial infarction in coronary artery disease patients with or without diabetes mellitus. Manuscript submitted.

Paper 3

Strand E, Bjørndal B, Nygård O, Burri L, Berge C, Bohov P, Christensen BJ, Berge K, Wergedahl H, Viste A, Berge RK (2012): Long-term treatment with the pan-PPAR agonist tetradecylthioacetic acid or fish oil is associated with increased cardiac content of n-3 fatty acids in rat. *Lipids Health Dis*. Jun 27;11(1):82.

Contents

1. Introduction

1.1 Coronary heart disease

Coronary heart disease (CHD) remains a major death cause based on reports from the American Heart Association [1]. Although mortality rates for CHD and acute myocardial infarction (AMI) have declined during the past three decades, as reported through "The Compressed Mortality database" containing mortality and population counts for all U.S. counties (http://wonder.cdc.gov/mortSQL.html), the overweight and obesity epidemic is increasing [2]. Obesity is one of the components of the metabolic syndrome [3] which is defined by having a minimum of three out of the following risk factors: abdominal obesity; impaired fasting glucose; elevated fasting triglycerides (TGs); decreased high density lipoprotein (HDL) cholesterol; or hypertension [4].

Established CHD therapy includes 3-hydroxy-3-methylglutaryl coenzyme A (CoA) reductase inhibitors (statins), acetylsalicylic acid, β-blockers, and angiotensin converting enzyme (ACE) inhibitors [5]. Statins are established as first-line treatment of hyperlipidemia [6], and this medication reduces cholesterol biosynthesis by inhibiting its rate-limiting enzyme, which subsequently leads to an increase in number of hepatic low density lipoprotein (LDL) receptors and thereby reduces the circulating levels of LDL and very low density lipoprotein (VLDL) through increased catabolism [7]. Effects are not only on plasma LDL cholesterol, but plasma TGs are also reduced, while plasma HDL cholesterol is increased. Several pleiotropic properties have been reported following statin treatment, including anti-inflammatory, vasodilatory, and anti-platelet effects [7]. Clinically, statin therapy leads to a moderate decrease in cardiovascular mortality, but a significant and more pronounced decrease in cardiovascular morbidity, with the largest benefit being suggested among intermediate/high-risk patients [5]. Dietary intakes of omega-3 (n-3) long-chain

polyunsaturated fatty acids (LCPUFAs) have been associated with a reduced risk of CHD mortality and are currently recommended by the American Heart Association in secondary prevention among CHD patients [8]. However, the 2012 European Society of Cardiology guidelines do not implement n-3 LCPUFA supplements as routine treatment among AMI patients with ST-segment elevation [9]. There is a continuous search for new drugs or dietary factors for improvement of lipid metabolism and most importantly for prevention of clinical events. Lifestyle habits like smoking, physical inactivity, and poor nutritional status are important factors to be considered in this context. These general risk factors can further culminate into conditions like dyslipidemia, overweight/obesity, metabolic syndrome, and diabetes mellitus that are major risk factors for development of cardiovascular diseases (CVD), including CHD.

1.2 Diabetes mellitus

Diabetes mellitus is a high-prevalent disease worldwide, where 90-95% of total cases are classified as type 2 diabetes [10]. This metabolic disorder develops as a result of an interaction between several factors [11], some based on genetic predisposition and some environmentally dependent. Altogether, poor nutrition and decreased physical activity increase the prevalence of lifestyle disorders, which might result in development of type 2 diabetes mellitus. Under healthy conditions, there is a feedback mechanism which signals the pancreatic β-cells for insulin secretion whenever needed [12]. During insulin resistance this mechanism might be disturbed and lead to β-cell dysfunction, which could result in diabetes development. A disturbed balance in adipose tissue, as seen in obese patients and in those with impaired glucose tolerance, increases the amount of plasma non-esterified fatty acids (FAs).

In addition to this increase in levels of non-esterified FAs, several metabolic pathways are affected in patients with diabetes, leading to hyperglycemia and sustained insulin resistance [10]. These conditions further affect molecular mechanisms which lead to vascular dysfunction, including increased oxidative stress and imbalance in

intracellular signaling. Common macrovascular consequences include atherosclerosis and medial calcification, while retinopathy and nephropathy are among microvascular manifestations.

Present recommendations on management of type 2 diabetes include the following [13]: individual guidance in terms of dietary advice and plasma glucose monitoring; regular monitoring of blood pressure and, when appropriate, treatment of hypertension; control and management of glycosylated haemoglobin (HbA1c) levels; assessment and management of cardiovascular risk factors; monitoring parameters relevant for kidney function; regular screening of eyes and the nervous system; and management of foot problems.

The disease primarily affects cardiovascular health [13], and chance of experiencing an AMI and also of a more severe outcome is increased in these patients compared to non-diabetic patients [14]. Thus, treatment and prevention of diabetes should be a priority, being a major risk factor for CVD and mortality [15].

1.3 Pathways affected by dietary fatty acids

Several molecular pathways are involved in dietary FA signaling, and regulation occurs at multiple levels, including cell surface receptor signaling (e.g. FA derived prostaglandins bind to G-protein coupled receptors), receptor-mediated pathways requiring FA acylation (e.g. G-protein, *Ras*, and *Src* kinase), intermediate signaling (e.g. through phospholipase C and protein kinase C), and nuclear receptor signaling [e.g. through peroxisome proliferator-activated receptors (PPARs)] [16].

The biological effects of polyunsaturated FAs (PUFAs) are exerted through several mechanisms including their release by cytosolic phospholipase A2 ($cPLA_2$), further being metabolized into eicosanoids (prostaglandins, thromboxanes, and leucotrienes) by the action of cyclooxygenases (COX) and lipoxygenases (LOX). While arachidonic acid (ARA) is the precursor of the 2-series prostanoids [e.g. prostacyclin

(PGI_2) and thromboxane A2 (TXA_2)] and leukotriene A4 (LTA_4), EPA is the precursor of the 3-series prostanoids [e.g. prostaglandin I3 (PGI_3) and thromboxane A3 (TXA_3)] and leukotriene A5 (LTA_5). n-3 PUFAs can also affect the production of the anti-inflammatory resolvins through separate pathways involving COX, LOX, and cytochrome P450 (CYP) [17]. Furthermore, free FAs can exert direct effects on ion channels and alter the Na^+ current and Ca^{2+} fluctuation. Membrane phospholipid composition affects both membrane fluidity and membrane-associated protein signalling. Effects on important pathways through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and extracellular signal-regulated kinases (ERK, activated through *Ras*) regulate gene transcription.

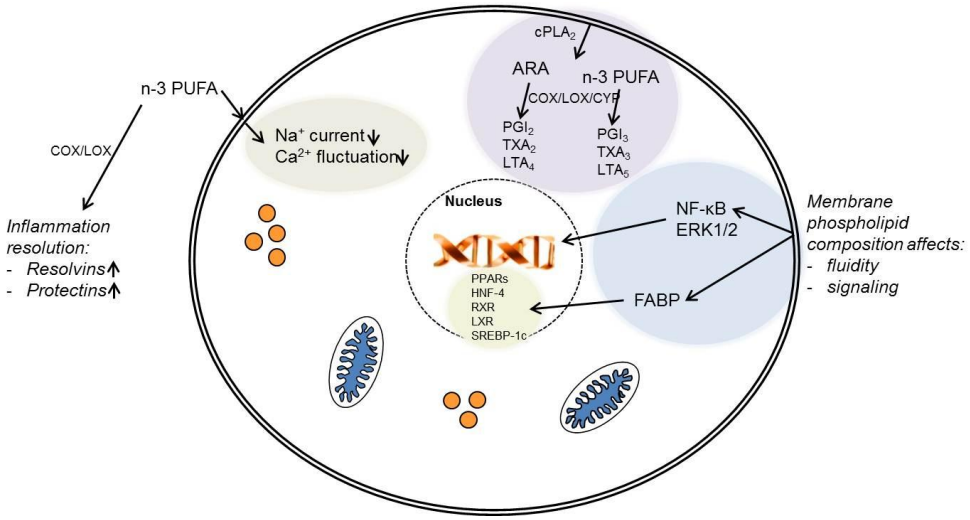

Figure 1. Pathways affected by dietary polyunsaturated fatty acids. Abbreviations: ARA, Arachidonic acid; COX, Cyclooxygenase; $cPLA_2$, Cytosolic phospholipase A2; CYP, Cytochrome P450; ERK, Extracellular signal-regulated kinases; FABP, Fatty acid binding protein; HNF-4, Hepatocyte nuclear factor 4; LOX, Lipoxygenase; LTA_4, Leukotriene A4; LTA_5, Leukotriene A5; LXR, Liver X receptor; n-3 PUFA, n-3 polyunsaturated fatty acids; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PGI_2, Prostacyclin; PGI_3, Prostaglandin I3; PPARs, Peroxisome proliferator-activated receptors; RXR, 9-*cis* retinoic acid receptor; SREBP-1c, Sterol regulatory element binding protein 1c; TXA_2, Thromboxane A2; TXA_3, Thromboxane A3.

Release of FAs into the cell and their association with fatty acid binding protein (FABP) can regulate important transcription factors like PPARs, hepatocyte nuclear factor 4 (HNF-4), 9-*cis* retinoic acid receptor (RXR), liver X receptor (LXR), and sterol regulatory element binding protein 1c (SREBP-1c) (**Figure 1**) [18].

In the following sections, focus will be on the PPAR mediated pathways, which are considered to be most relevant for this thesis.

1.3.1 Peroxisome proliferator activated receptors

PPARs are members of the nuclear hormone receptor family consisting of three subtypes (α, γ, and β/δ) with distinct and overlapping expression patterns [19]. These receptors are ligand-activated transcription factors which heterodimerize with RXR before targeting peroxisome proliferator-response elements (PPREs) on DNA, and thereby regulate transcription of several target genes which finally affect lipid metabolism, glucose homeostasis, and cell differentiation [20]. The result of activation depends on PPAR tissue distribution, ligands, and presence of co-activators and -repressors. Natural ligands of PPAR include both saturated and unsaturated non-esterified FAs [21]. PPARs have been designated with the role as central regulators of interactions between genes and diet [22], and have become pharmaceutical targets for treatment of conditions related to dyslipidemia.

PPARα activation targets several genes connected to glucose and lipid metabolism, including FA uptake, -transport, and -oxidation, and its expression is especially high in liver, heart, and kidney [23]. It is also involved in embryonic development [24] and amino acid metabolism [25]. Rodents, such as mice and rats, belong to the proliferating species, meaning that PPARα activation causes peroxisome proliferation in liver [26]. The so-called non-proliferating species, like guinea pigs, pigs, monkeys, as well as humans, have a lower PPARα expression in liver [27]. In the heart PPARα seems to be the primary transcription regulator of enzymes involved in FA oxidation and is thus important for cardiac metabolism [28]. PPARα operates together with PPARγ co-activator (PGC-1α) [29]. In addition to being expressed in vascular

endothelial cells, monocytes/macrophages, and smooth muscle cells, PPARα is also expressed in inflammatory cells related to atherosclerosis [30]. The healthy heart generates most of its energy as ATP through FA catabolism [31]. Thus, a continuous FA supply to the heart is important to sustain the contractile activity as this tissue can store and synthesize FAs only to a limited extent [32]. Excess PPARα stimulation may, however, have detrimental effects [33] including substrate overload of FAs in the tissues, which is also associated with conditions like obesity and insulin resistance [34]. Myocardial dysfunction could hereby be the result of an obesity-associated reduced glucose and increased FA utilization in heart [35,36]. Whereas hepatic PPARα activity is mainly regulated by endogenous FAs, cardiac PPARα activity is regulated by exogenous fatty acids. Lipolysis from cellular TGs is necessary to enable exogenous FAs as potent cardiac PPAR ligands through adipose TG lipase (ATGL). If ATGL function is impaired, FAs will be stored in the form of TGs instead of being oxidized and lead to increased levels of cardiac neutral lipids [37]. This might further affect mitochondrial function through altered protein phosphorylation and increased levels of cytotoxic intermediate products of β-oxidation, which could lead to mitophagy (controlled degradation of mitochondria) or apoptosis [38].

PPARγ is highly expressed in adipose tissue and is involved in adipocyte differentiation and lipid storage [39]. Activation also contributes to improved insulin sensitivity [20] and anti-inflammatory properties [40]. This subtype has been detected in cardiomyocytes in several species [41], but probably holds an indirect modulatory role on cardiac FA metabolism [42]. PUFAs do more preferentially bind to PPARγ than to PPARα. Despite this, they are not very potent PPARγ activators [21]. Other endogenous PPARγ agonists are eicosanoids and components of oxidized LDL [30]. Synthetic PPARγ agonists, like thiazolidinediones and glitazones, are used in treatment of patients with type 2 diabetes [43].

Compared to PPARα and PPARγ, PPARβ/δ is more ubiquitously expressed [20]. Expression is particularly pronounced in the intestine, compared to other tissues. In rodents, this PPAR subtype is expressed predominantly in cardiomyocytes [44].

Stimulated β-oxidation and reverse cholesterol transport are among the processes affected by activating this receptor [24]. Among PPARβ/δ natural ligands are 6-18 C saturated FAs (SFAs), some PUFAs, prostacyclins, and FAs derived from VLDLs [30]. There are also several synthetic ligands with preference to PPARβ/δ [45], but none are at present in clinical use [20].

PPAR agonists relevant for this thesis, which are mainly associated with PPARα activation, will be discussed in the sections to follow.

1.3.2 PPAR agonists

Polyunsaturated fatty acids

Linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) are essential FAs in the mammalian diet, unable to be endogenously synthesized [46]. Dietary sources which are rich in LA are corn oil, sunflower oil, and safflower oil, while ALA is abundant in flaxseeds and flaxseed oil as well as some other oils and nuts [47]. Fish and fish oil (FO) is rich in the n-3 LCPUFAs eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), which are poorly oxidizable [48]. Current dietary recommendations are to increase intake of n-3 PUFAs and reduce intake of n-6 PUFAs, since CHD mortality has been shown to be proportional to tissue n-6 PUFA composition [49]. The adverse effects resulting from a high intake of n-6 PUFAs are related to an increase in n-6 eicosanoid action, involved in developing vascular inflammation, thrombosis, and arrhythmia.

PUFAs are natural ligands for all PPARs [46], and they also affect additional nuclear receptors that modulate TG levels, including HNF-4α [21]. As for PPARγ, PUFAs are not very strong PPARα activators, but eicosanoids and oxidized FAs which are metabolites of PUFAs are more potent activators [50,51]. The TG lowering effects of PUFAs (especially n-3 PUFAs) are attributed to PPAR activation and increased FA oxidation, downregulated HNF-4α and induced glycogen synthesis, and suppression of hepatic lipogenesis by inhibition of SREBP-1c [21]. Altogether, these effects

reduce TG storage and increase oxidation of FAs. In addition, n-3 PUFAs have been shown to reduce VLDL secretion through degradation of apolipoprotein B (Apo B) [52], as well as improving chylomicron clearance [53].

Fibrates

Fibrates are a group of specific PPARα targeted drugs being utilized in treating dyslipidemia during the past four decades due to their TG reducing effects [54]. TG lowering is probably obtained through a PPARα activated increase in VLDL and chylomicron TG hydrolysis. Fibrates also lower circulating non-esterified FAs, increase HDL cholesterol, and modestly lower LDL cholesterol levels. They induce transcription of genes necessary for FA cellular uptake and have also been suggested to reduce vascular inflammation [54]. Their clinical gain, however, still remains to be demonstrated [55,56], and their routine use in combined hyperlipidemia has recently been questioned [57]. Even though overall use of fibrates in treatment of CVD has failed to demonstrate additional benefits compared to conventional statin treatment, some effects have been shown in sub-groups like diabetes patients. Benefits of treatment using the fibrate gemfibrozil were more pronounced in insulin resistant patients with a larger reduction in cardiovascular events compared to those without insulin resistance [58]. In a study on patients with type 2 diabetes, fenofibrate treatment lead to reduced angiographic progression of atherosclerosis [59].

Notably, treatment with fibrates has never been associated with reduced incidence of CVD death. Thus, excess PPARα activation may be associated with unfavorable metabolic effects that may counteract its apparent beneficial effects on lipid metabolism and inflammation. In the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial, fenofibrate treatment was associated with reduced risk of non-fatal AMI and coronary revascularization [60]. However, plasma homocysteine was increased following fenofibrate treatment. Elevated homocysteine has been considered as a biomarker of increased CVD risk [61], and a post hoc sub-study based on the FIELD trial investigating this effect demonstrated that increased homocysteine levels were directly associated with levels of apolipoprotein A-II (Apo A-II) in

fenofibrate allocated patients [62]. The role of Apo A-II is not completely characterized, but it seems to be involved in TG metabolism and has recently been associated with apolipoprotein E (Apo E)-linked risk for incident CVD [63].

Tetradecylthioacetic acid

Tetradecylthioacetic acid (TTA) is an SFA analogue with 16 carbon atoms and one sulphur atom at position three from the carboxyl end (**Figure 2**), belonging to a group of sulphur-substituted FAs (3-thia FAs) with pan-PPAR activation properties [64]. This modified FA is a mitochondrial targeted compound with properties similar to n-3 PUFAs and an especially pronounced affinity to PPARα, similar to fibrates. In addition, TTA has a moderate affinity to PPARβ/δ and a weak affinity to PPARγ [65,66,67]. Due to the presence of a sulphur atom, TTA is unable to undergo β-oxidation. This modified FA is known to reduce plasma TGs, probably due to hepatic proliferation of mitochondria and an increased β-oxidation of FAs through PPAR-dependent mechanisms [68]. Also, feeding-induced obesity is prevented and insulin sensitivity improved in hyperlipidemic models following TTA administration [69]. TTA has effects that are similar to fibrates in type 2 diabetic patients, as demonstrated through an open-label study, where participants received 1 g TTA daily for four weeks, resulting in reduced serum LDL cholesterol [70].

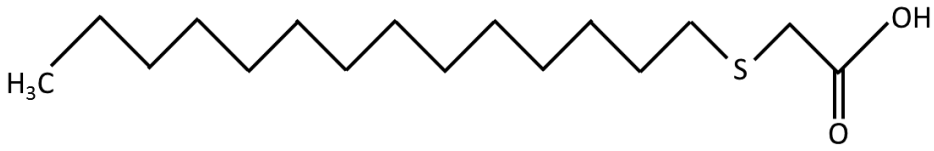

Figure 2. Structure of tetradecylthioacetic acid (TTA).

1.4 Metabolism and heart disease

1.4.1 Fatty acid metabolism

FAs destined for the heart mainly comes from albumin-bound, adipose tissue derived, non-esterified FAs or from TG components of VLDL or chylomicrons [71]. TG components are hydrolysed into non-esterified FAs by lipoprotein lipase (LPL). FAs are the primary energy substrate for the heart which are delivered to cardiomyocytes via a three protein-mediated mechanism (cluster of differentiation 36, FA transport protein, and FA binding protein plasma membrane) [72,73], and acyl-CoA esters are formed following cell entry by acyl-CoA synthetases, processes which are regulated by PPARs [74]. Esterified FAs are destined to various fates: incorporation into TG or plasma membrane phospholipids following elongation and desaturation; complete FA catabolism and energy production; or conversion to derivatives like eicosanoids via the COX or LOX pathways [46]. The essential FAs LA and ALA are the precursors for longer chain n-6 and n-3 PUFAs which are important constituents of cell membranes [75].

Fatty acid synthesis

Endogenous FAs are synthesized through a series of steps involving elongases and desaturases (**Figure 3**) [76]. Δ9 desaturase is involved in the conversion of SFAs into monounsaturated FAs (MUFAs), introducing a double-bond at the 9, 10 position of FAs. The membrane-bound Δ6 and Δ5 desaturases introduce additional double-bonds in n-9, n-6, and n-3 PUFAs [77]. As n-6 and n-3 PUFAs compete for the same desaturases and elongases, the dietary intakes of these FAs are essential for which pathway will be prioritized. Thus, FA composition of cell membranes partly depends on the dietary intake of essential FAs. In addition to dietary intake, the overall FA status also depends on endogenous synthesis and transport, controlled by several factors including genetics, age, sex, extent of oxidative stress, and lifestyle [78].

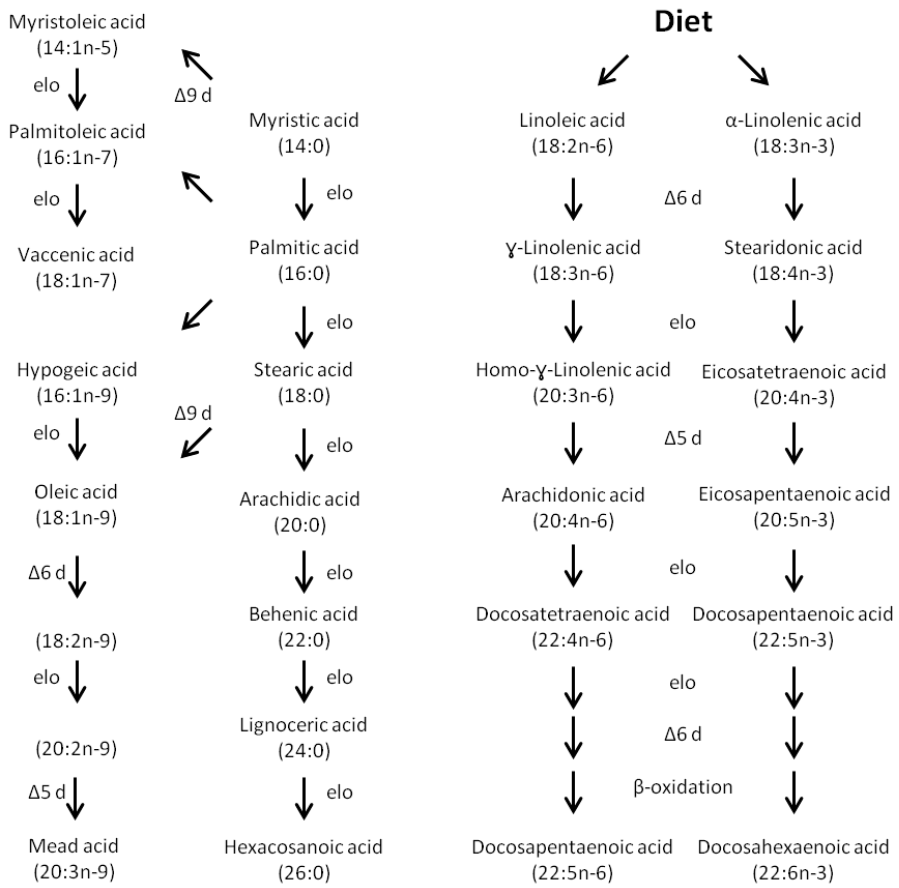

Figure 3. Fatty acid metabolism. Abbreviations: Δ9 d, Δ9 desaturase; Δ6 d, Δ6 desaturase; Δ5 d, Δ5 desaturase; elo, elongase. A total overview of fatty acid metabolism is complex. Thus, this simplified illustration includes only fatty acids that are considered relevant for this thesis.

Fatty acid catabolism

Mammalian tissues most commonly depend on mitochondrial pyruvate decarboxylation (from glucose, lactate, or amino acids) or FA oxidation (especially of long-chain FAs) to provide energy [79]. Some FAs are dependent on the peroxisomes to undergo oxidation, especially very long-chain FAs. Other FAs can be metabolized in either peroxisomes or mitochondria, while short-chain FAs are exclusively

oxidized in mitochondria [80]. Typically, FAs destined for peroxisomes undergo the first step(s) of oxidation in this organelle, generating a number of acyl-CoA esters which are subsequently transported for further oxidation in mitochondria.

Carnitine maintains a rigorous balance between free and esterified CoA in the cell, and is present in cells and tissues as free L-carnitine and acylcarnitines of a wide variety of chain-lengths [81]. L-carnitine is a water-soluble quaternary amine that is endogenously synthesized from lysine and methionine, but can also be obtained through the diet [82,83]. The carnitine shuttle is involved in transport of long-chain FAs into the mitochondrial matrix for β-oxidation. Carnitine palmitoyltransferase I (CPT-I) is a PPARα regulated protein located in the outer mitochondrial membrane which form long-chain acylcarnitine esters (>C12) through transesterification, where acyl-groups from acyl-CoA are transferred to L-carnitine. CPT-I is regarded as a key regulator of mitochondrial β-oxidation of long-chain FAs, shuttling acylcarnitines across the mitochondrial membranes with the help of carnitine-acylcarnitine translocase (CACT). A second carnitine palmitoyltransferase, CPT-II, present in the mitochondrial matrix, catalyzes the transesterification to intramitochondrial CoA. Free L-carnitine can leave the mitochondria via CACT. Carnitine acetyltransferase (CRAT), which is present both in the mitochondrial matrix and in peroxisomes, has the ability to reconvert short- and medium-chain acyl-CoAs to acylcarnitines [84], which can also leave the mitochondria through CACT and be transported out of the tissue to other destinations or for urinary excretion [79]. Complete mitochondrial β-oxidation generates acetyl-CoA, a process which is also regulated by PPARα. Acetyl-CoA is further metabolized in the tricarboxylic acid (TCA) cycle, ultimately generating energy through the electron transport chain [74].

1.4.2 Triglycerides and cardiovascular disease

Increased levels of TGs have been associated with CVD risk, and for this reason numerous approaches have been made to achieve reduced TG levels. TG synthesis is situated in the hepatocytes, regulated by SREBP-1c. Substrates for TG synthesis are

glucose and non-esterified FA. Glycolysis provides pyruvate, from which acetyl-CoA is made available for entering the TCA cycle, and finally conversion of citrate to TGs. Non-esterified FAs are directly incorporated into TGs [21].

Particles rich in TGs are VLDL, LDL, intermediate density lipoprotein (IDL), and chylomicrons [85]. Human TGs usually have SFAs and MUFAs as building blocks, with n-3 PUFA in only trace amounts. Approaches to improve insulin sensitivity are probably the best way to decrease TGs, which could be achieved by increased physical activity and weight loss [86]. Furthermore, TG levels can be reduced by drugs like statins, fibrates, TTA, and thiazolidinediones, but also by high-dose dietary n-3 LCPUFAs [85]. After several years studying TG and its association with CVD, one can conclude that TG is a relatively weak risk factor in itself. However, it is a marker of underlying changes related to lipid- and lipoprotein metabolism [85].

Since dietary n-3 LCPUFAs have a well-known effect of reducing plasma TG, hypertriglyceridemia has been one of the main indications for n-3 LCPUFA treatment related to CHD [87].

1.4.3 Omega-3 fatty acids and cardiovascular disease

In general, consumption of fish and n-3 LCPUFAs, mainly EPA and DHA, has been associated with a reduced risk of CVD and mortality [88,89]. The most likely mechanism of action is the antiarrhythmic properties of EPA and DHA [90]. In addition to their association with reduced TG levels, dietary intake of n-3 LCPUFAs has also been related to anti-inflammatory effects [91,92]. Beneficial effects seem particularly pronounced in patients with reduced ventricular function and heart failure [93]. At the molecular level, dietary n-3 LCPUFA alters cardiac mitochondrial phospholipid composition, thereby affecting mitochondrial function [94], and exerts pleiotropic effects considered to be cardioprotective [95]. Altogether, EPA and DHA phospholipid incorporation is increased on the expense of reduced ARA content. Very high doses of n-3 PUFA can, however, be pro-oxidative [96].

Existing international guidelines recommends an n-3 LCPUFA intake of at least 250 mg/day or 2 servings of oily fish per week for the general population [97]. The recommended intake for patients with CVD is 1 g/day and for hypertriglyceridemic patients 2-4 g/day [8]. In Norway, and especially in coastal Western Norway, fish consumption and supplemental intakes of n-3 LCPUFAs have traditionally been high [98,99], and only few people would be expected to have intakes below the suggested threshold. No clinical benefits were seen among Norwegian patients with previous AMI who received 4 g/day of n-3 LCPUFA supplements for 1-2 years, although TG levels were significantly decreased following n-3 LCPUFA consumption [100]. Similarly, there was no reduction in total cardiovascular events or mortality in Danish patients with chronic hemodialysis receiving 1.7 g/day of n-3 LCPUFAs [101]. However, the number of incident AMIs was significantly reduced in this high-risk population. In a Norwegian trial among elderly men with high-risk of CVD, there was a borderline statistically significant reduced risk of all-cause mortality associated with an increased intake of n-3 LCPUFAs [102]. Two Japanese studies showed no association between a higher intake of n-3 LCPUFAs and coronary artery disease (CAD) mortality or sudden death [103,104]. There was, however, a significantly decreased risk of non-fatal coronary events, mainly AMI, with high intakes of n-3 LCPUFAs. Even though the overall associations between n-3 LCPUFA intakes and reduced risk of major cardiovascular events and mortality are controversial, there are indications of beneficial effects on certain outcomes with intakes above the suggested threshold level.

1.4.4 Omega-3 fatty acids and diabetes mellitus

Numerous studies have focused on diets among patients with diabetes, with conflicting evidence and inconclusive results regarding its associations to dietary n-3 LCPUFAs [105,106,107,108,109,110]. Although several studies have demonstrated associations between increasing intakes of n-3 PUFAs and decreased incidence of diabetes [111,112,113], a large cohort study in US adults without pre-existing chronic disease [114] and a prospective study in women [115] concluded that high intakes of

n-3 LCPUFAs and fish might increase the incidence of type 2 diabetes. A study in post-MI patients did not report any relations between n-3 LCPUFAs and cardiovascular events [116]. However, a post hoc analysis among the diabetic participants revealed a strong decline in cardiovascular events after n-3 PUFA supplementation [117].

In the diabetic state, cardiac metabolism is modified by systemic metabolic changes, which alters lipid profile and thus FA utilization. This could eventually result in severe disease such as heart failure [118]. Randomized trials in heart failure patients have demonstrated reduced mortality [119] and improved left ventricular systolic function and functional capacity [93,120] following n-3 LCPUFA intervention. Previous studies indicate insulin resistance among patients with heart failure [121]. Data from studies in persons with type 2 diabetes have shown benefits of n-3 LCPUFAs on blood TG, with no improvement in insulin sensitivity or glucose control [87]. Treatment strategies including dietary intake of n-3 LCPUFA have been recommended among patients with type 2 diabetes according to the American Heart Association and the American Diabetes Association statements from 2007 [122].

2. Aims of study

2.1 Overall aim

The overall aim was to investigate the dietary intake of n-3 LCPUFAs and risk of future coronary events in patients with CAD and to explore possible mechanistic effects of PPAR activation in heart by bioactive FAs in an animal model.

2.2 Specific aims

1. To examine the relation between dietary intake of n-3 LCPUFAs or fish and risk of incident coronary events or mortality in a patient cohort having well-characterized and well-treated CAD (90% statin users) and a relatively high consumption of n-3 LCPUFAs.

2. To reveal a possible influence of impaired glucose metabolism on n-3 LCPUFA effects by studying the association between n-3 LCPUFA intake and risk of AMI in CAD patients with or without diabetes mellitus. This was based on the beneficial effects previously observed in heart failure patients.

3. To study PPAR mechanisms in the rat heart by investigating the long-term influence of the poorly oxidizable mitochondrial targeted pan-PPAR agonists TTA and/or high-dose FO on cardiac lipid metabolism. The effect on FA composition and related gene expression was observed in an animal model. Data from liver was applied to reveal possible organ specific effects in the heart.

3. Subjects and Methods

3.1 Human studies

3.1.1 Study population

The participants in the human studies included in this thesis were a sub-selection of patients included in the Western Norway B-Vitamin Intervention Trial (WENBIT). The main study was a prospective, randomized, double-blind, placebo-controlled secondary prevention study which investigated the effect of homocysteine lowering B vitamins (0.8 mg folic acid + 0.4 mg vitamin B-12), vitamin B-6 (40 mg), their combination, or placebo on cardiovascular outcomes and all-cause mortality [123]. Eligible patients in the trial were men and women aged >18 years undergoing coronary angiography for suspected CAD and/or aortic valve stenosis at Haukeland University Hospital or Stavanger University Hospital in Western Norway. Patients who participated in other trials, abused alcohol, suffered from mental illness, had cancer, or were unavailable for follow-up were excluded from participating in the trial. Patients were recruited between 1999 and 2004. During this period a total of 10241 patients underwent coronary angiography for suspected CAD or acute coronary syndrome (ACS) at the two study centers. Due to capacity reasons, not all eligible patients were screened. A total of 4241 patients at Haukeland University Hospital consented to withdrawal of blood at baseline, regardless of them being included in WENBIT after coronary angiography. This total cohort is denoted the Bergen Coronary Angiography Cohort (BECAC). At Stavanger University Hospital 969 patients were included in WENBIT, with blood samples being withdrawn exclusively in those being recruited into the study after diagnosed with CAD assessed by coronary angiography. Thus, a biobank was built up of samples from a total of 5210 patients from the two hospitals, denoted the Western Norway Coronary Angiography Cohort (WECAC). All participants who were eligible for and consented to participate in

BECAC signed a consent form, and participants in WENBIT signed a separate consent form. A total of 3090 patients were randomly assigned into the WENBIT study. The study protocol was in accordance with the principles of the Declaration of Helsinki, and the trial was approved by the Regional Committee for Medical Research Ethics, the Norwegian Medicines Agency, and the Data Inspectorate. Overall, there was no short- or long-term benefits on cardiovascular outcome associated with the study treatment [123].

In **Paper 1** we studied participants in WENBIT who completed a semiquantitative food-frequency questionnaire (FFQ) at trial enrollment. In total, 2484 patients completed the FFQ. Nineteen questionnaires were excluded because they contained more than one blank page. Participants with very low (<3000 kJ for women and <3300 kJ for men) or very high (>15000 kJ for women and >17500 kJ for men) estimated daily energy intakes were excluded (n=53), leaving 2412 patients with valid dietary data. In **Paper 2** we investigated the same cohort as in **Paper 1**, but by doing sub-group analyses according to parameters of glucose metabolism, and 34 patients were excluded because of missing HbA1c data. Thus, **Paper 2** included a total of 2378 patients with available data on HbA1c and with FFQ considered of adequate quality. **Figure 4** provides an overview of patient selection from baseline collection of blood samples and recruitment to WENBIT, and to final inclusion in the sub-studies presented in **Paper 1** and **2**. In an independent study based on the source population for patients recruited to WENBIT at Haukeland University Hospital, serum FA composition was determined among 1367 consecutive patients examined in 2000-2001. In the currently investigated cohort, 723 patients had FA composition data.

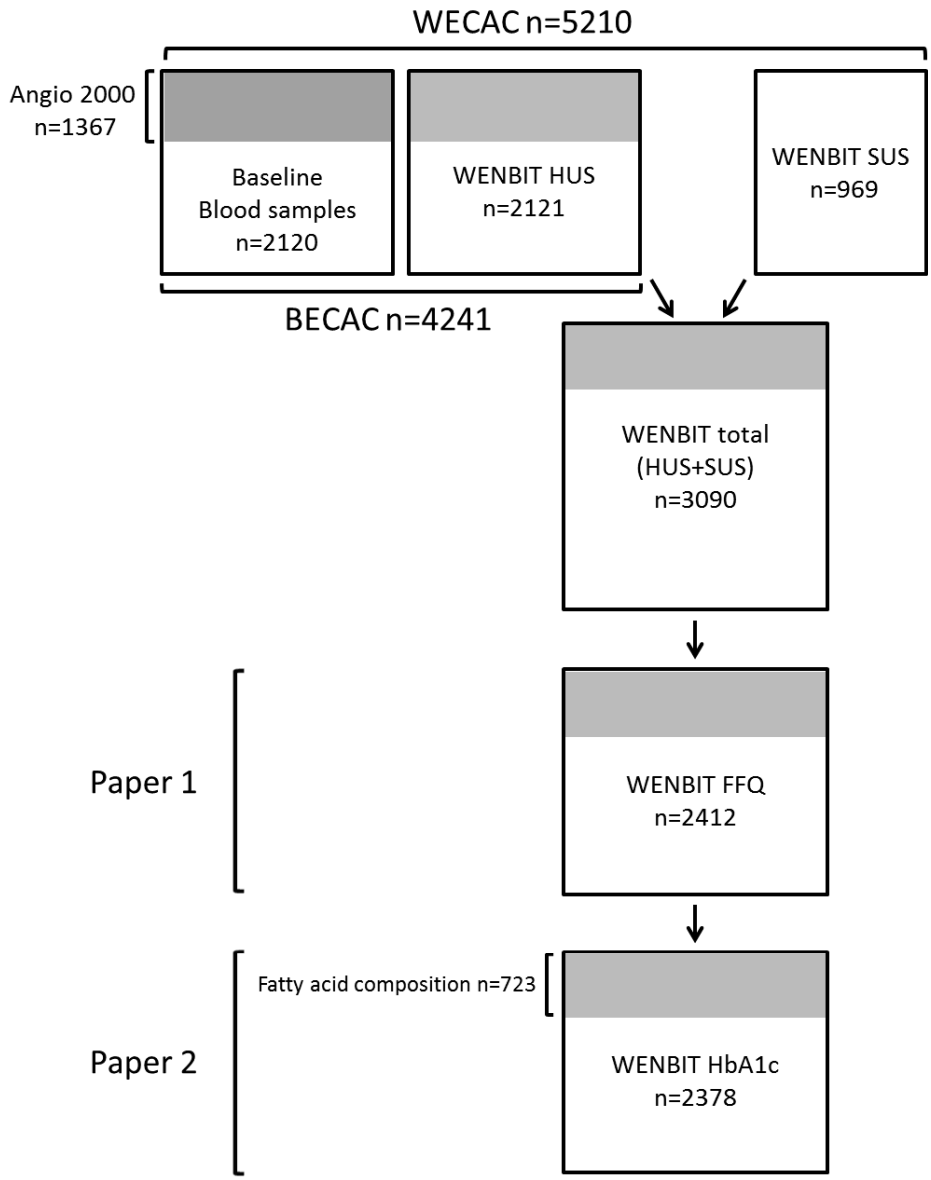

Figure 4. Flow of randomized patients from WECAC to study populations in Papers 1 and 2. WENBIT FFQ designates the study population in **Paper 1** and WENBIT HbA1c the study population in **Paper 2**. Abbreviations: WECAC, Western Norway Coronary Angiography Cohort; WENBIT, Western Norway B-Vitamin Intervention Trial; HUS, Haukeland University Hospital; SUS, Stavanger University Hospital; BECAC, Bergen Coronary Angiography Cohort; FFQ, Food Frequency Questionnaire.

3.1.2 Dietary assessment

The FFQ (**Supplement A**) was developed at the Department of Nutrition, University of Oslo, and had previously been validated against plasma phospholipid FA concentrations in adult and older Norwegian men and women with correlations of 0.51 and 0.49, and the ability to classify 81% and 78% into the same or adjacent quartile, for EPA and DHA, respectively [124]. The FFQ was given to patients on the day of enrollment and returned by mail to the study center or collected at the follow-up appointment 1 month later. The FFQ included 169 food items that were grouped according to Norwegian meal patterns and was designed to obtain information on usual food intake during the past year. The frequency of consumption was given per day, week, or month, depending on the items in question. The portion sizes were given as household measures or units such as slices or pieces. Intakes of fish and fish products were assessed by questions related to breakfast, lunch, and dinner. For breakfast or lunch, amounts were estimated from number of sandwiches with the following spreads eaten per week: tinned mackerel in tomato paste or smoked mackerel; sardines, pickled herring, anchovies, or similar; and salmon or trout (11 frequency categories). For lunch or dinner, categories were fish cakes, fish pudding, or fish balls; fish fingers; boiled cod, coalfish, or haddock; fried cod, coalfish, or haddock; fresh, salt-cured, or smoked herring; fresh or smoked mackerel, salmon, or trout (wild or farmed); fish stew, fish soup, or fish au gratin; and shrimp or crab (9 frequency categories and 5 related amount categories such as piece, fillet, or household measures). Dietary fish data was presented as total fish intake. The FFQ also included questions about supplements, including cod liver oil, cod liver oil capsules, and FO capsules. Intake categories were 3-fold: whole year or winter use only, times per week, and amount per time. Total intake of n-3 LCPUFA was used for the analyses. Nutrient intake was calculated by using a database and a software system developed at the Department of Nutrition, University of Oslo (Kostberegningssystem, version 3.2; University of Oslo, Norway) [125].

3.1.3 Assessment of clinical data

Demographic, clinical, and routine laboratory data were obtained by study personnel. Smokers included self-reported current smokers, those reported having quitted within the last four weeks, and patients with plasma cotinine levels ≥85 nmol/L. Left ventricular ejection fraction (LVEF) (%) was determined by ventriculography or echocardiography and values <50% were considered to be impaired. Estimated glomerular filtration rate (eGFR) was calculated applying the Chronic Kidney Disease Epidemiology Collaboration [126]. In **Paper 2**, participants were sub-grouped into non-diabetic (no previous diabetes and HbA1c <5.7%), pre-diabetic (no previous diabetes and HbA1c ≥5.7%), and diabetic (previously diagnosed diabetes or fasting baseline serum glucose ≥7.0 or a non-fasting glucose ≥11.1 mmol/L). These threshold levels were selected based on established numbers for diagnosing pre-diabetes and diabetes [127]. According to the new diabetes definitions, patients with HbA1c ≥6.5% are classified with diabetes. It is presently unknown if this patient group hold a risk of macrovascular complications which is comparable to those with diabetes either clinically diagnosed or defined by threshold glucose levels [128]. Therefore patients with HbA1c ≥6.5% who were not previously diagnosed with diabetes were classified as having pre-diabetes in our study.

3.1.4 Assessment of laboratory data

Standard blood laboratory parameters were analyzed from fresh samples according to routine protocols at the respective central laboratories at the two study centers. Samples for the biobank were collected together with routine blood samples at baseline coronary angiography and stored at -80˚C until analysis. Reagent kits of type Tina-quant® on Apolipoprotein A-I (Apo A-I, ver.2), Apo B (ver.2), and C-reactive protein (latex, high sensitive assay) were obtained from Roche Diagnostics (GmbH, Mannheim, Germany), and serum measurements on these parameters were done on the Hitachi 917 system (Roche Diagnostics). HbA1c was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF

MS) [129] and plasma cotinine by liquid chromatography/tandem mass spectrometry (LC/MS/MS) at BEVITAL AS (http://www.bevital.no). Serum FA methyl esters (FAMEs) were obtained by heating of lipids with methanol at 90°C for one hour. Sulphuric acid was used as a catalyst [130]. After extraction into an organic solvent, FAMEs were analyzed by gas-liquid chromatography (GC). The gas chromatograph (GC 8000 TOP, Finnigan, Austin, TX, USA) was equipped with a programmed temperature vaporization injector, flame-ionization detector, AS 800 autosampler, and with a fused silica capillary column DB1-ms (J & W Scientific, Folsom, CA, USA). Hydrogen was used as a carrier gas. Column temperature was programmed from 110 to 310°C with a gradient of 2.5°C/min. GC signal was acquired and evaluated with Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA). Peaks were identified by means of known FA standards and by means of mass spectra, obtained by GC/MS analysis (GCQ, Finnigan) on the same column. Internal standard (C21:0) was used for quantification after calibration with known mixtures of FA standards. LDL cholesterol was calculated by using the Friedewald formula [131].

3.1.5 Endpoints and follow-up

The main endpoint in **Paper 1** was a composite of coronary events comprising hospitalization for unstable angina pectoris, non-fatal AMI, and coronary death. In addition, the following separate endpoints were considered: all-cause death, coronary death, AMI (fatal and non-fatal), and stable angina pectoris with angiographically verified progression of CAD. In **Paper 2**, the endpoint was fatal and non-fatal AMI, presented both as total AMI and as separate fatal and non-fatal AMIs.

Events and supplemental medical information were collected from hospitals and on deaths from the Norwegian Cause of Death Registry. If death occurred ≤28 days after the onset of an event, the event was classified as fatal. AMI was classified according to the diagnostic criteria of the revised definition of AMI from 2000 [132]. Procedure-related non-fatal AMI occurring ≤24 hours after coronary angiography, percutaneous coronary intervention (PCI), or coronary artery bypass graft surgery

(CABG) were excluded. Cases of unstable angina pectoris were classified as endpoints if patients were urgently admitted to hospital due to acute attacks of typical ischemic symptoms, accompanied by electrocardiographic ST–T findings of myocardial ischemia at rest, and/or if coronary angiography verified significant progression of their CAD [133]. Endpoints were recorded until December 31st 2006, and all events were adjudicated by members of the endpoints committee.

3.1.6 Statistical analyses

Paper 1

In **Paper 1**, participants were ranked into quartiles of n-3 LCPUFA consumption [combined daily intakes of EPA, docosapentaenoic acid (DPA, 22:5n-3), and DHA] expressed as percentage of total energy (%TE), to control for confounding by differences in energy intake [134], or ranked into quartiles of fish intake (g).

Statistical analyses were performed by using SPSS for Windows, version 15 (SPSS Inc, Chicago, IL) and R version 2.0 (The R Foundation for Statistical Computing, Vienna, Austria). Generalized additive models (GAMs) were applied to explore any non-linear associations between intake of n-3 LCPUFAs and the main endpoint of coronary events after adjustment for potential confounders [135,136]. The functional form of the natural logarithm of n-3 LCPUFA intake (g) was modeled with a smoothing spline fit (4 df) in a multivariate Cox proportional hazards model. Power was assessed on the basis of a 2-sided chi-square test (significance level of 5%) comparing quartile 1 with quartiles 2–4 (combined) (SamplePower 2.0; SPSS Inc, Chicago, IL). The statistical power to detect a decrease in event rate from 15% in quartile 1 to 10% in quartiles 2–4 of n-3 LCPUFAs (33% decrease in relative risk) was 90%. To detect a decrease from 15% to 12% (20% decrease in relative risk) would give a power of 46%.

Paper 2

In **Paper 2**, participants within each sub-group of non-diabetes, pre-diabetes, and diabetes were ranked into tertiles of daily n-3 LCPUFA (%TE) and fish (g) intake.

Spearman's rank correlation was used to assess associations between various continuous parameters. The Kolmogorov-Smirnov test was used to examine the continuous FA variables for normal distribution. Variables that were not normally distributed were log-transformed. Estimated marginal means and 95% confidence intervals (CIs) of FA profile were calculated for non-diabetic, pre-diabetic, and diabetic participants by one-way analysis of covariance (ANCOVA), with adjustments made for age, sex, and statin dose. Post-hoc comparisons for specified between-group differences were made by using the Tukey HSD test for FAs where groups significantly differed as assessed by ANCOVA.

Survival curves were created for follow-up until the 95th percentile of follow-up time (corresponding to 6.8 years) using the Kaplan-Meier method. Interactions between intake of n-3 LCPUFAs and diabetes were tested by adding product terms in the Cox model. Statistics were performed by using IBM SPSS Statistics for Windows, version 19 (SPSS Inc., Chicago, IL, USA) and R version 2.15.2 (R Development Core Team, Vienna, Austria).

Paper 1 and 2

Means and SDs [or medians (25th, 75th percentile)] and proportions were computed for selected baseline characteristics and dietary variables. Trends across quartiles or tertiles were tested by using linear regression for continuous variables and logistic regression for binary variables.

Hazard ratios (HRs) and 95% CIs were estimated by using Cox proportional hazards. Tests for trend were performed by assigning equally spaced weights for each quartile (1–4) or tertile (1-3) of n-3 LCPUFA or fish intakes and modeling this as a

continuous variable in separate Cox proportional hazards models. The basic model included age and sex. Additional covariates in the multivariate model were selected on the basis of clinical relevance (**Table 1**).

Table 1. Covariates included in the multivariate adjusted models in Cox regression analyses in Papers 1 or 2

	Continuous	Categorical	Paper 1	Paper 2
Hypertension		Yes or no	X	
Current smoking		Yes or no[1]	X	X
Serum triglycerides	mmol/L			X
Left ventricular ejection fraction	%		X	X
Extent of CAD		0-3[2]		X
Current use of statins		Yes or no	X	
Acute coronary syndrome		Yes or no	X	X
Diabetes mellitus		Yes or no	X	
Baseline PCI		Yes or no		X
Baseline CABG		Yes or no		X
Fasting		Yes or no		X
Folic acid treatment		Yes or no		X
Vitamin B6 treatment		Yes or no		X

Abbreviations: CABG, coronary artery bypass graft surgery; CAD, coronary artery disease; PCI, percutaneous coronary intervention

[1]Still smoking at baseline or <1 month since quitting, or assessed by cotinine levels ≥85 nmol/L

[2]Non-significant; single, double, or triple vessel

Several covariates were assessed for adjustment, but did not appreciably alter the results and were not included in the final model (**Table 2**). Physical activity was not included because data was missing in 23% of the patients. *P*-values <0.05 were considered to be statistically significant.

Table 2. Covariates assessed for use (but not included) in Cox regression analyses in Papers 1 or 2

	Continuous	Categorical	Paper 1	Paper 2
Body mass index		Quartiles	X	X
Previous AMI		Yes or no	X	
Previous PCI		Yes or no	X	
Previous CABG		Yes or no	X	
Previous cerebrovascular disease		Yes or no	X	
Previous carotid artery stenosis		Yes or no	X	
Previous peripherial arterial disease		Yes or no	X	
Serum apolipoprotein A-I	g/L			X
Serum apolipoprotein B	g/L			X
Glycosylated haemoglobin	%			X
C-reactive protein	mg/L			X
Current use of β-blockers		Yes or no	X	X
Current use of ACE inhibitors		Yes or no	X	X
Current use of metphormin		Yes or no		X
Current use of sulphonamides		Yes or no		X
Current use of insulin		Yes or no		X
Dietary intake of SFA		Quartiles	X	
Dietary intake of n-6 PUFA		Quartiles	X	
Dietary intake of ALA		Quartiles	X	
Dietary intake of fiber		Quartiles	X	
Dietary intake of thiamine		Quartiles	X	
Dietary intake of riboflavin		Quartiles	X	
Dietary intake of tocopherol		Quartiles	X	
Physical activity		0-3[1]	X	X

Abbreviations: ACE, angiotensin-converting enzyme; ALA, α-linolenic acid; AMI, acute myocardial infarction; CABG, coronary artery bypass graft surgery; CAD, coronary artery disease; n-6 PUFA, n-6 polyunsaturated fatty acids; PCI, percutaneous coronary intervention; SFA, saturated fatty acids

[1] 0, 1, 2-3, or ≥4 days/week

3.2 Animal study

3.2.1 Study design and sampling

Male Wistar rats, aged eight to ten weeks, were obtained from Taconic Europe A/S. They were housed in groups of five and maintained at a constant 12 hours light-dark cycle at a temperature of 22 ± 1ºC and a relative humidity of 55 ± 5%. Animals were acclimatized under these conditions for one week prior to study start and had free access to standard chow during the acclimation period and water at all times. During the feeding period the animals received one out of five diets for a period of 50 weeks (**Table 3**).

Table 3. Animal study diets

Diet (% w/v)[1]	Low fat	Control	TTA	FO	TTA + FO
Lard (fat)	5.0	23.0	22.6	12.6	12.2
Soybean oil (fat)	2.0	2.0	2.0	2.0	2.0
TTA			0.375		0.375
EPAX 4020 TG				10.4	10.4
Casein (protein)	15.6	19.7	19.7	19.7	19.7
Cornstarch	57.2	35.3	35.3	35.3	35.3
Sucrose	10.0	10.0	10.0	10.0	10.0
Fiber	5.0	5.0	5.0	5.0	5.0
AIN-93G mineral mix	3.5	3.5	3.5	3.5	3.5
AIN-93 vitamin mix	1.0	1.0	1.0	1.0	1.0
L-cysteine	0.3	0.3	0.3	0.3	0.3
Choline bitartrate	0.25	0.25	0.25	0.25	0.25
Tert-butyl-hydroquinone	0.014	0.0014	0.0014	0.0014	0.0014
KH_2PO_4, monobasic	0.13				

Abbreviations: TTA, tetradecylthioacetic acid; FO, fish oil; EPAX 4020 TG, fish oil provided by EPAX AS

[1]Ingredients are given in mass concentration (mass/volume, % w/v)

After 50 weeks, the animals were sacrificed using isoflurane (Forene, Abbott Laboratories, Abbott Park, IL) under non-fasting conditions. The abdomen was opened in the midline and blood was drawn by cardiac puncture and collected in BD Vacutainer tubes containing EDTA (Becton, Dickinson, and Company, Plymouth, UK). The heart and liver tissues were collected and immediately freeze-clamped as

drainage of blood from the animal was complete. Plasma and tissue samples were stored at -80˚C until analyses.

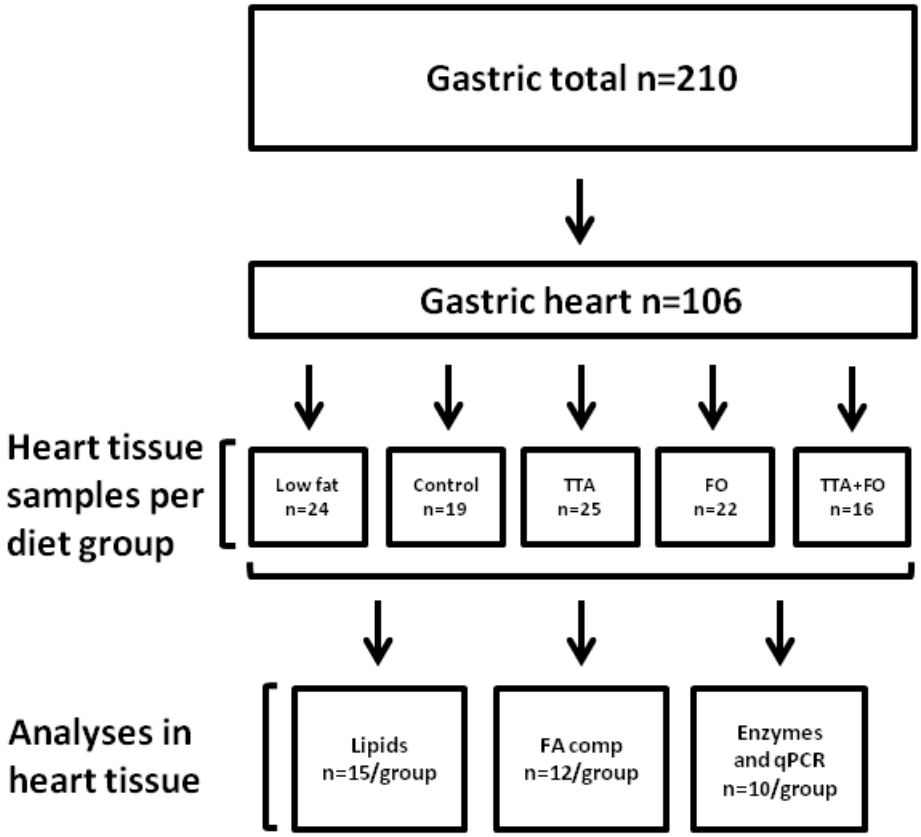

Figure 5. Sub-group analyses on heart tissue of male Wistar rats after dietary intervention with TTA and/or FO – Gastric heart study. A total of 210 rats were included in the main study, from which 106 were selected for heart tissue sampling. Number of available tissue samples in each group and number of animals included in each analysis were as designated. Abbreviations: TTA, tetradecylthioacetic acid; FO, fish oil; FA comp, fatty acid composition; qPCR, real-time polymerase chain reaction.

A total of 210 animals were included in the main study. As this study was not solely designed for heart tissue analyses, the heart was only dissected out in 106 animals. Between 10 and 15 heart tissue samples from each diet group was selected for the following analyses (**Figure 5**): lipid pattern; FA composition; enzyme activity; or

real-time polymerase chain reaction (qPCR). Selections were made based on availability of heart tissue, capacity/cost, and achieving a sufficient sample size.

The animal experiments were standardized according to the Guidelines for the Care and Use of Experimental Animals, and the protocol was approved by the Norwegian State Board for Biological Experiments with Living Animals.

3.2.2 Tissue analyses

Tissue samples were homogenized and lipids extracted with chloroform-methanol [137]. Samples were evaporated under nitrogen and re-dissolved in isopropanol before lipid analysis on the Hitachi 917 system (Roche Diagnostics, GmbH, Mannheim, Germany). Total cholesterol (CHOD-PAP) and TG (GPO-PAP) kits were from Roche Diagnostics and the phospholipids kit from DiaSys Diagnostic Systems GmbH (Holzheim, Germany). FAMEs were obtained from tissue extracted lipids and FA composition was analyzed by following the same procedure as for serum, which was described in **Section 3.1.4**.

After homogenization and fractionation [138], enzymatic activities of CPT-I and -II [139], fatty acyl-CoA oxidase (ACOX) [140,141], glycerol-3-phosphate acyltransferase (GPAT) [142], and FA synthase (FAS) [143] were measured in the post-nuclear extracts of heart tissue.

Total cellular RNA was purified by using the RNeasy kit and the protocol for fibrous tissue (Qiagen GmbH, Hilden, Germany). Prior to gene analyses, RNA quantity was determined spectrophotometrically (NanoDrop 1000, NanoDrop products, Wilmington, DE, USA), while quality was evaluated by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies Inc., Santa Clara, CA, USA). RNA was reversely transcribed to cDNA in 100 µl reactions using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). Samples were treated with RNase inhibitors as part of the protocol. Selected genes were analyzed using qPCR (ABI PRISM 7900 HT Sequence Detection System, Applied

Biosystems): Rn00566242 (*Cpt-Ib*), Rn00571166 (*Ucp2*), Rn00565874 (*Ucp3*), Rn00580241 (*Pgc1α*), Rn00580051_m1 (*Tfam*), Rn01455958_m1 (*Nrf1*), Rn00580728 (*Cd36*), Rn00588652 (*Cact*), Rn00577366 (*Fabp3*), Rn00563649 (*Acadvl*), Rn00566390 (*Acadm*), Rn00574634 (*Acads*), Rn00566193 (*Pparα*), Rn00565707 (*Pparδ*), Rn00440945 (*Pparγ*), and Rn00585821_m1 (*Fatp1*). All primer/probe sequences for the studied genes were obtained from Applied Biosystems. The MIQE guidelines for qPCR analyses were used when selecting house-keeping genes [144,145].

3.2.3 Statistical analyses

The results were presented as means with their standard deviations (SD) for a minimum of eight and a maximum of fifteen rats per group. Gene expression data was normalized against the control diet group. The low-fat diet group was excluded from the analyses, as this group was not comparable to the TTA- and FO-intervention groups. Data was evaluated by two-way analysis of variance (ANOVA) for treatment additivity and synergy [146]. Results were not adjusted for multiple comparisons, and thus *P*-values <0.01 were considered significant. Statistics were performed by using PASW Statistics for Windows, version 18 (SPSS Inc., Chicago, IL, USA).

3.3 Methodological considerations

3.3.1 Human studies

The studied cohort (**Paper 1 and 2**) was large and well-characterized with high accuracy in data collection including angiographic examination of CAD and a long-term follow-up with respect to clinical endpoints. Being a prospective cohort study among patients with stable angina, participants were likely to have stable dietary habits, which might provide a more correct picture of sustained dietary intake during follow-up compared with patients suffering an ACS. On the other hand, results obtained in this patient cohort do not automatically apply to the general population. Notably, since most previous studies have based their results on circulating levels of n-3 LCPUFAs and since many studies have been done on patient groups not receiving statins, data is scarce on the association between dietary intake of n-3 LCPUFA and AMI in patients with CAD who are treated with statins. Our study had limited power to detect significant effects due to a lower event rate than expected, and for this reason we cannot exclude the possibility of false negative (type II error) or positive (type I error) results. This is particularly applicable for the non-diabetes group in **Paper 2**.

Observational studies like the current investigation are frequently applied in order to: validate results from randomized controlled trials in less selective populations; investigate sub-groups of interest; and generate new hypotheses as a basis for further studies [147]. Since the exposure variables in this cohort were measured at baseline only, while the outcome events occurred up to several years after, it is possible that the exposure status might have changed during this period, resulting in a regression dilution bias [148]. In order to correct for this, we would have needed multiple measurements over time. Thus, we cannot rule out that estimates might have been biased and thus the effects could be under- or overestimated. By studying characteristics in the total population of FFQ respondents and non-respondents as described in **Paper 1**, it seemed that patients with stable angina both had the most

frequent FFQ response rate and the highest n-3 LCPUFA intake. Thus, since these were patients with mostly stable angina and already established CAD, it can be assumed that any changes in dietary habits due to known heart disease had already been established at study start [149]. However, increased intakes of n-3 LCPUFAs combined with other lifestyle factors like physical activity would altogether affect the results. For this reason, we cannot exclude reverse causality or confounding from inappropriately clarified or unrevealed risk factors or lifestyle habits in this study, despite careful adjustments for important covariates. As these patients were all participants in WENBIT, a B-vitamin intervention trial, it was necessary to rule out the possibility of treatment with folic acid/vitamin B12 or vitamin B6 as potential confounders. Adjustments for B-vitamin intervention in the survival analyses did not affect the outcome. Furthermore, 89% of the patients were treated with statins. A modifying effect by statins on n-3 LCPUFA supplementation has previously been investigated, where no additional benefits could be found on top of statin treatment [150]. Although we cannot rule out such an effect modification, adjustment for the effective dose of statin treatment (expected % reduction in LDL of the actual type and dose of statin) did not change the outcome (data not shown).

While selection bias might be a potential problem in observational studies, randomized controlled trials are designed to eliminate such bias. However, when observational studies are properly designed there seem to be no major differences in outcome as compared to related randomized controlled trials [151]. Whereas randomized controlled trials are typically limited by ethical rules to avoid major adverse effects, observational studies do not have the same ethical challenges. The current study was performed in a cohort with extensive data collection and follow-up, reducing the confounding/selection bias to a minimum. AMI was chosen as the main endpoint of the sub-group analysis in **Paper 2**, although the outcome-rate was overall low. Using a composite endpoint as in **Paper 1** increases the number of events. However, it has been stated that composite endpoints might in general be inadequate, since the results apply to the individual components of the endpoint [152].

The use of sub-group analyses may be contentious. Although there may be limited reliability of sub-group analyses in general, due to a risk of bias, we have important reasons for performing such analyses in this prospective study: (1) A solid clinical hypothesis, supported by internal and external evidence and (2) Plausible pre-defined sub-groups directly related to the hypotheses and aims. We did not perform multiple testing additional to these hypotheses, in line with articles discussing this topic [153,154].

Due to resource constraints, FFQs were not checked for errors when received at the study center, and participants with extreme values or partially missing reported intakes were excluded ahead of the current studies. However, the estimated dietary intakes were comparable to previous surveys in the region using the same questionnaire [155].

Serum was used for the FA analyses. Within-day coefficient of variation did not exceed 5% for any FA, making the analyses accurate. In principle, serum FA profile reflects the dietary intake corresponding to the latest meal before blood sampling [46]. Thus, measuring parameters like FA composition and HbA1c during follow-up would have been very useful to confirm that a similar pattern could be detected. Unfortunately, due to cost and capacity reasons, this was not prioritized. Altogether, any changes in patient status, like diet, lifestyle, and medications during follow-up, would potentially influence the outcome. For optimization of FA composition results, analyses might have been performed in erythrocytes because of their slower turnover rate compared to lipoproteins in serum. Thus, erythrocyte samples have been suggested to more likely resemble the long-term FA intake as compared to its serum values [156]. Furthermore, we did not have serum FA composition data on the entire study population. Thus, risk assessment was based on dietary data. Moreover, since the majority of the patients in the studied cohort used statins, which significantly influence the FA profile in plasma [157], FA composition was adjusted according to statin dose before presenting the results.

3.3.2 Animal study

The animals in this thesis were part of a larger study [158], which included a jejuno-gastric reflux surgical procedure. A separate experiment enduring 11 weeks were done comparing animals with and without operation, to make sure that the procedure did not affect the nutritional state in the animals. There was no difference in body weight or plasma lipids between the two groups (data not shown), and thus it was assumed that the operation had no adverse effects regarding nutrition.

The following house-keeping genes were included in gene analyses: Hs99999901_s1 (*18S*, Eurogentec S.A., Seraing, Belgium), Rn99999916_s1 (*Gapdh*, Applied Biosystems), and Rn00821065_g1 (*Arbp*, Applied Biosystems). The *18S* house-keeping gene was found to be the most consistent between the diet groups using geNorm [159] and was selected for normalizing the expression value of each gene in all samples.

4. Main results

4.1 Paper 1

In this study among 2412 patients with CAD, mean age was 61.7 years, and 80.5% were men. There were 84.7% with stable angina pectoris, 14.1% with ACS, and 1.3% with aortic valve stenosis at baseline angiography. The majority of participants received first-line treatment with acetylsalicylic acid and statins.

Mean (± SD) daily dietary intakes of n-3 LCPUFAs and total fish across quartiles of n-3 LCPUFA (footnote 1) or total fish (footnote 2) intake were as shown in **Table 4**. Intakes of oily fish, as well as FO and cod liver oil supplements, increased across quartiles of n-3 LCPUFA intake.

Table 4. Daily dietary intakes by quartiles

	Quartile 1	Quartile 2	Quartile 3	Quartile 4
n-3 LCPUFAs (%TE)[1]	0.15 ± 0.06[3]	0.34 ± 0.06	0.57 ± 0.08	1.15 ± 0.40
n-3 LCPUFAs (g)[1]	0.58 ± 0.29	0.83 ± 0.30	1.36 ± 0.44	2.64 ± 1.18
Total fish (g)[1]	64.3 ± 40.3	100.4 ± 48.0	123.9 ± 54.1	149.8 ± 84.6
Total fish (g)[2]	41.1 ± 16.3	81.4 ± 9.3	118.0 ± 12.4	198.0 ± 63.8

Abbreviations: n-3 LCPUFAs, n-3 long-chain polyunsaturated fatty acids; %TE, percentage of total energy

[1]By quartiles of n-3 LCPUFA intake (%TE)

[2]By quartiles of total fish intake (g)

[3]Mean ± SD (all such values)

During a median (5th, 95th percentiles) follow-up of 57 (33, 83) months 292 patients experienced a coronary event, 137 patients died (76 cases were coronary death), 210 had an AMI, and 298 patients with stable angina had an angiographic verified progression of their CAD.

Table 5. Hazard ratios (and 95% CIs)[1] for coronary events by quartiles of n-3 long-chain polyunsaturated fatty acids (LCPUFAs) (%TE) and fish (g)

Intake of	n-3 LCPUFA[2]	Total fish[3]	Lean fish[4]	Oily fish[5]	Processed fish[6]
Age- and sex-adjusted					
Quartile 1	1.00	1.00	1.00	1.00	1.00
Quartile 2	0.80 (0.58, 1.12)	1.04 (0.75, 1.45)	0.80 (0.57, 1.12)	0.65 (0.46, 0.92)	0.88 (0.64, 1.21)
Quartile 3	0.86 (0.62, 1.19)	1.04 (0.75, 1.44)	0.99 (0.72, 1.36)	1.00 (0.74, 1.36)	0.78 (0.57, 1.08)
Quartile 4	0.93 (0.68, 1.28)	0.98 (0.70, 1.36)	0.92 (0.67, 1.28)	0.92 (0.67, 1.27)	0.83 (0.60, 1.14)
P for trend	0.77	0.88	0.95	0.78	0.19
Multivariate[7]					
Quartile 1	1.00	1.00	1.00	1.00	1.00
Quartile 2	0.82 (0.59, 1.14)	1.08 (0.78, 1.50)	0.79 (0.56, 1.11)	0.66 (0.47, 0.94)	0.88 (0.64, 1.20)
Quartile 3	0.90 (0.65, 1.24)	1.07 (0.77, 1.48)	1.00 (0.73, 1.37)	1.00 (0.74, 1.37)	0.77 (0.56, 1.07)
Quartile 4	0.95 (0.69, 1.31)	1.04 (0.74, 1.45)	0.98 (0.70, 1.36)	0.95 (0.69, 1.31)	0.86 (0.63, 1.19)
P for trend	0.89	0.86	0.76	0.69	0.26

[1]Hazard ratios and 95% CIs were calculated by using Cox proportional hazards.
[2]Combined eicosapentaenoic-, docosapentaenoic-, and docosahexaenoic acids.
[3]Total of lean-, oily-, processed-, and unspecified fish, in addition to shellfish and fish sandwich.
[4]Combined cod, pollock, and haddock.
[5]Combined herring (included pickled), mackerel (included smoked and in tomato sauce), salmon, trout, sardines, anchovies, or similar.
[6]Combined fish cakes, fish pudding, fish balls, fish sticks, fish stew, fish soup, and fish gratin.
[7]Multivariate model adjusted for age (continuous), sex, left ventricular ejection fraction (continuous), diabetes mellitus (yes or no), hypertension (yes or no), current smoker (still smoking at baseline or <1 month since quitting), acute coronary syndrome (yes or no), and current use of statins.

The age- and sex-adjusted risk of having a coronary event was 20%, 14%, and 7% lower for patients in quartiles 2, 3, and 4 of n-3 LCPUFAs, respectively, compared to the lowest quartile, without any significant or dose-response relations (**Table 5**). When comparing quartiles 2–4 (combined) with quartile 1 there was a 13% risk reduction (P=0.27). HRs were not appreciably changed after multivariate adjustments.

There were no associations between intake of n-3 LCPUFAs and all-cause or coronary mortality or AMI. Furthermore, there was no association between n-3 LCPUFA intake and risk of verified progression of CAD in patients with stable angina. A post hoc additive proportional hazards model indicated a statistically

significant increased risk at the very lowest end, equivalent to a daily intake of n-3 LCPUFAs below approximately 300 mg.

No associations could be seen between fish intake (total-, lean-, oily-, or processed fish in g/day) and coronary events (**Table 5**) or any other studied outcome. Similarly, risk of coronary events did not differ between users and non-users of FO or cod liver oil.

4.2 Paper 2

The study population comprised 2378 patients with HbA1c data, where 16 (0.7%) had type 1 and 301 (12.7%) type 2 diabetes, either clinically diagnosed or determined according to fasting baseline serum glucose ≥7.0 or a non-fasting glucose ≥11.1 mmol/L before angiography. This study cohort had very similar characteristics to the one in **Paper 1**, being the same cohort except from 34 patients who were excluded due to missing HbA1c data. Mean (± SD) daily dietary intakes of n-3 LCPUFAs, including supplements, among all 2378 participants were 0.43 ± 0.24, 1.08 ± 0.37, and 2.38 ± 1.15 g/day for tertiles 1-3, respectively.

Serum FA profiles were evaluated among 723 patients. There was a strong association between intake and serum levels of total n-3 LCPUFAs (rho=0.515, $P<0.001$). There was no difference in mean serum levels of total or individual n-3 LCPUFAs between the three sub-groups.

During a mean (± SD) follow-up of 4.8 (± 1.4) years, a total of 208 participants (8.7%) experienced a fatal or non-fatal AMI. Intake of n-3 LCPUFAs was energy adjusted and given as %TE in the analyses.

In sub-groups, 1012 patients were classified with non-diabetes, 1049 patients with pre-diabetes, and 317 patients with diabetes. In non-diabetic patients there was a non-significant trend towards an increased risk of experiencing an AMI with n-3 LCPUFA intakes corresponding to the upper vs. lower tertile. No associations were seen

between tertiles of n-3 LCPUFA consumption and risk of AMI in pre-diabetic patients, and restricting the analysis to patients with HbA1c ≥6.5% (n=423) provided similar results (**Table 6**). Among patients with diabetes there was a significantly reduced risk of experiencing an AMI in the upper vs. lower tertile of n-3 LCPUFA intakes (*P*=0.02), with a dose-response relation (*P* for trend=0.01). Adding patients with HbA1c ≥6.5% to the diabetes group clearly weakened the results (**Table 6**).

Table 6. Hazard ratios (and 95% CIs) for acute myocardial infarction (fatal and non-fatal) by tertiles of dietary n-3 LCPUFA (%TE)[1]

	423 patients with HbA1c ≥6.5%[2]		740 patients with diabetes or HbA1c ≥6.5%[3]	
	Age/sex adjusted	**Multivariate[4]**	**Age/sex adjusted**	**Multivariate[4]**
Number of events	32		75	
Tertile 1[5]	1.00	1.00	1.00	1.00
Tertile 2[6]	1.26 (0.55, 2.89)	1.24 (0.53, 2.86)	0.83 (0.49, 1.42)	0.93 (0.53, 1.61)
Tertile 3[7]	0.86 (0.35, 2.11)	0.91 (0.36, 2.28)	0.66 (0.38, 1.16)	0.71 (0.40, 1.26)
P for trend	0.74	0.85	0.15	0.24

[1]LCPUFA, long-chain polyunsaturated fatty acids; %TE, percentage of total energy. Hazard ratios and 95% CIs were calculated by using Cox proportional hazards.
[2]Sub-group defined as those with HbA1c ≥6.5%, but with a fasting glucose <7.0 or a non-fasting glucose <11.1 mmol/L and no previously diagnosed diabetes.
[3]Sub-group defined as clinically diagnosed diabetes, having a fasting glucose ≥7.0 or a non-fasting glucose ≥11.1 mmol/L, or having HbA1c ≥6.5%.
[4]Multivariate model adjusted for age (continuous), sex, fasting (yes or no), current smoker (yes or no), extent of coronary artery disease (non-significant; single, double, or triple vessel), left ventricular ejection fraction (continuous), triglyceride levels (continuous), baseline acute coronary syndrome (yes or no), baseline percutaneous coronary intervention (yes or no), baseline coronary artery bypass graft surgery (yes or no), and treatment with folic acid or vitamin B6 supplements (yes or no).
[5]The number of patients in tertile 1 of n-3 LCPUFA was 141 in the sub-group with a total of 423 patients and 246 in the sub-group with a total of 740 patients.
[6]The number of patients in tertile 2 of n-3 LCPUFA was 141 in the sub-group with a total of 423 patients and 247 in the sub-group with a total of 740 patients.
[7]The number of patients tertile 3 of n-3 LCPUFA was 141 in the sub-group with a total of 423 patients and 247 in the sub-group with a total of 740 patients.

Also, a separate analysis including the 258 patients who were previously clinically diagnosed with diabetes, regardless of baseline blood glucose levels, did not change the result (**Table 7**). The diabetes sub-group tended to have an increased n-3

LCPUFA intake compared to the others, and thus analysis was repeated using total population based tertiles, providing similar results (**Table 7**).

Table 7. Hazard ratios (and 95% CIs) for acute myocardial infarction (fatal and non-fatal) by tertiles of dietary n-3 LCPUFA (%TE)[1]

	Diabetes (n=258)[2]		Diabetes (n=317)[3]	
	Age/sex adjusted	**Multivariate[4]**	**Age/sex adjusted**	**Multivariate[4]**
Number of events	36		43	
Tertile 1[5]	1.00	1.00	1.00	1.00
Tertile 2[6]	0.55 (0.26, 1.17)	0.62 (0.27, 1.42)	0.53 (0.25, 1.11)	0.61 (0.27, 1.36)
Tertile 3[7]	0.33 (0.14, 0.80)	0.32 (0.13, 0.81)	0.48 (0.23, 0.97)	0.50 (0.23, 1.07)
P for trend	0.01	0.02	0.04	0.07

[1]LCPUFA, long-chain polyunsaturated fatty acids; %TE, percentage of total energy. Hazard ratios and 95% CIs were calculated by using Cox proportional hazards.
[2]Diabetes defined as clinically diagnosed.
[3]Diabetes defined as clinically diagnosed, or as having a fasting glucose ≥7.0 or a non-fasting glucose ≥11.1 mmol/L, with n-3 LCPUFA tertiles based on the total study population (n=2378).
[4]Multivariate model adjusted for age (continuous), sex, fasting (yes or no), current smoker (yes or no), extent of coronary artery disease (non-significant; single, double, or triple vessel), left ventricular ejection fraction (continuous), triglyceride levels (continuous), baseline acute coronary syndrome (yes or no), baseline percutaneous coronary intervention (yes or no), baseline coronary artery bypass graft surgery (yes or no), and treatment with folic acid or vitamin B6 supplements (yes or no).
[5]The number of patients in tertile 1 of n-3 LCPUFA was 86 in the sub-group with a total of 258 patients and 98 in the sub-group with a total of 317 patients.
[6]The number of patients in tertile 2 of n-3 LCPUFA was 86 in the sub-group with a total of 258 patients and 99 in the sub-group of 317 patients.
[7]The number of patients in tertile 3 of n-3 LCPUFA was 86 in the sub-group with a total of 258 patients and 120 in the sub-group of 317 patients.

When doing separate analyses on fatal- and non-fatal AMIs there was an almost 5-fold significantly increased risk of fatal AMI in the upper vs. lower tertile of n-3 LCPUFA intakes (*P* for trend=0.02) in non-diabetic patients, but a significant reduction by 78% in the upper vs. lower tertile (*P* for trend=0.02) in patients with diabetes. There were no significant risk associations for non-fatal AMI in any sub-group.

Interestingly, in the non-diabetic sub-group mean (± SD) HbA1c levels were significantly lower in tertiles 2 (*P*=0.008) and 3 (*P*=0.01), compared to tertile 1 of n-3

LCPUFA intakes (4.87 ± 0.62 and 4.87 ± 0.65 vs. 4.99 ± 0.54, respectively). Furthermore, non-diabetic patients who experienced an AMI had significantly lower mean HbA1c levels, compared to those who did not experience an AMI (4.77 ± 0.63 vs. 4.92 ± 0.60, P=0.04). This association was strengthened and more pronounced among those who had a fatal AMI event (4.55 ± 0.68 vs. 4.92 ± 0.60, P=0.02). No such differences were observed in those with pre-diabetes or diabetes.

4.3 Paper 3

At study start, mean (± SD) weight of the animals was 266 (±32) g. Animals treated with TTA gained less weight during the study, compared to those not receiving TTA (P<0.001). After 50 weeks of follow-up, no changes could be seen in cardiac TG after TTA or FO treatment. There were significantly increased levels of total cardiac cholesterol after TTA treatment (mean ± SD, 3.21 ± 0.20 vs. 2.93 ± 0.24 μmol/g heart tissue, p<0.001) and of cardiac phospholipids after FO treatment (14.68 ± 0.91 vs. 13.69 ± 0.88 μmol/g heart tissue, p<0.001), compared to control.

There were several changes in both cardiac and hepatic FAs following TTA and/or FO administration. In heart, total FAs and mead acid (MA, C20:3n-9) were significantly increased, while total SFAs and ARA were decreased after TTA treatment. MUFAs and MA were significantly decreased following FO treatment. The n-3/n-6 PUFA ratio, as well as EPA and DHA, was significantly increased after TTA and FO treatment. Cardiac DPA were increased following TTA treatment and decreased following FO treatment. FA measurements in liver showed a significant increase in C16:0, total MUFAs, and MA, and a decrease in C18:0 and total n-6 PUFAs, after dietary intervention with TTA. Total SFAs, total MUFAs, MA, and total n-6 PUFAs were decreased in liver of rats treated with FO. In contrast to the increase in cardiac n-3 PUFAs, these FAs were significantly decreased in liver following TTA treatment, but increased after FO treatment.

Changes in cardiac activity of key metabolic enzymes were associated with TTA treatment, including reduced activity of CPT-I and increased activities of CPT-II, ACOX, GPAT, and FAS. Enzyme activities of ACOX and GPAT were also significantly increased by FO treatment.

Cardiac expression of several PPAR-targeted genes was affected by the intervention. *Cpt-Ib* (isoform of *Cpt-I* expressed in muscle), *Ucp3* (encoding the uncoupling protein 3), and *Cact* mRNAs were upregulated, while *Ucp2*, *Pparδ*, and *Pparγ* mRNAs were downregulated after TTA administration. *Cpt-Ib* and *Fatp1* were upregulated after FO administration. All above-mentioned effects of TTA and/or FO intervention on FA composition, enzyme activities, and gene expression were significant at the 1% level by two-way ANOVA.

5. Discussion

The main focus of this thesis was to study the associations between dietary intake of n-3 LCPUFAs and effects on coronary outcomes in humans. Effects of bioactive FAs were also studied in an animal model to get into more depth at the mechanistic level. First, we investigated possible relations between dietary n-3 LCPUFAs and coronary events in patients with established and well-treated CAD who had relatively high intakes of fish and n-3 LCPUFA supplements, without being able to reveal an overall association between intakes and events (**Paper 1**). Only patients with very low intakes seemed to have a slightly increased risk of experiencing a coronary event. Second, we aimed at elucidating possible effects in sub-groups of the original cohort from **Paper 1**, with a focus on patients with or without diabetes mellitus (**Paper 2**). We revealed a reduced risk of AMI in the upper, compared to the lower tertile of n-3 LCPUFA intakes among patients with diabetes. On the contrary, a high intake of n-3 LCPUFAs among non-diabetic patients with HbA1c levels <5.7% was associated with an increased risk of fatal AMI, although the event rate was limited in this sub-group. Third, since PUFAs are PPAR agonists and there have been much recent focus on PPAR targeted therapy in relation to CVD, we sought to discover the specific myocardial effects in a rodent model to explore possible mechanisms (**Paper 3**). Rats were treated with the pan-PPAR agonist TTA (having a main effect on PPARα) or high-dose FO (having effects on both PPARα and PPARγ), which after 50 weeks induced marked changes in cardiac PUFA composition resulting in a cardiac-specific increase in n-3 LCPUFA levels. Also, changes in enzyme activities and gene expression, which could be related to PPAR effects, indicated an increased cardiac FA oxidation.

5.1 Dietary intake of Omega-3 fatty acids and coronary events

Dietary intake of n-3 LCPUFAs and their proposed beneficial effects on cardiovascular- and coronary events has been a major focus among both researchers and "common people" during the recent decades. Conflicting results have been reported and the effects are under continuous debate. We found no associations between n-3 LCPUFA intake and coronary events or mortality in the total study cohort of patients with CAD (**Paper 1**). The mean intake of n-3 LCPUFAs was 1.35 g/day, and only 1.7% had intakes <250 mg/day. The observed trend of an increased risk of experiencing a coronary event among subjects with an intake below approximately 300 mg/day of n-3 LCPUFAs could indicate that patients with very low intakes of these FAs might benefit from increasing their fish- or n-3 LCPUFA supplementary intakes. This is in line with previous findings demonstrating reduced coronary death among those consuming fish once or twice a week, compared to those who rarely eat fish, as observed in some prospective cohort studies [160].

The current observations of a weak effect of n-3 LCPUFAs in patients with stable angina are consistent with findings from several studies, and recent meta-analyses have concluded similarly [161,162]. Other meta-analyses have demonstrated an association between a high dietary intake of n-3 LCPUFAs and reduced risk of cardiovascular death, cardiac death, all-cause mortality, and cardiovascular- and coronary events [163,164], although they partly included the same studies as the meta-analyses reporting no effects. It all seems to depend on the heterogeneity of the study populations, the inclusion criteria of the meta-analyses (population, dosage, and follow-up time), the type of statistical methods being used, and how results have been interpreted. Altogether, based on the large number of conflicting reports discussing a proposed effect of dietary n-3 LCPUFAs, it seems that no clear associations can be drawn for a wide, heterogeneous population of patients with or in high risk of CVD. Therefore, we further investigated possible associations in specific sub-groups within the study cohort. Based on studies in heart failure patients, where promising

beneficial effects have been demonstrated [93,119,120], we aimed at focusing more specifically at patients with diabetes and AMI incidence.

In some previous investigations, a high intake of fish or n-3 LCPUFAs have been associated with a reduced risk of non-fatal AMI [104,134,165,166]. A sub-group analysis of the large clinical trial JELIS concluded that there was an overall increased risk of experiencing a major coronary event among patients with impaired glucose metabolism, compared to normoglycemic patients, but that treatment with EPA significantly reduced the increased event rate in those with impaired glucose metabolism [167]. The association between a high intake of n-3 LCPUFAs and reduced risk of incident AMI among patients with diabetes as revealed in the current study (**Paper 2**) was especially pronounced for fatal AMI events. This may be an important finding related to secondary treatment of diabetes, bearing in mind the generally increased risk of cardiovascular mortality in this patient group [15]. The recent large randomized controlled trial (ORIGIN) in 12536 patients with dysglycemia and at high risk for cardiovascular events was unable to reveal any beneficial effects of daily n-3 LCPUFA supplementation [168]. Baseline dietary intake of n-3 LCPUFAs among participants in this trial was only about 200 mg/day, which was comparable to the intake in the very lowest spectre of our population. The intervention group received 1 g/day of n-3 LCPUFAs, while intakes in the upper tertile in our diabetes group had a mean intake of 2.42 g/day. Furthermore, participants in ORIGIN had a median HbA1c of 6.4% and fasting plasma glucose levels ≥6.1 mmol/L. In the current study, the diabetes group had a median HbA1c of 7.2%, and the fasting serum glucose threshold for diagnosing diabetes was ≥7.0 mmol/L. Notably, there was increased mortality after aggressive glucose lowering treatment in a study among diabetic patients with baseline median HbA1c 8.1% [169]. Thus, an overall intensive glucose lowering in ORIGIN may also have influenced outcome following n-3 LCPUFA supplementation. Based on our results demonstrating no effect in the pre-diabetes group or in the separate analyses where all patients with HbA1c ≥6.5% were grouped together with patients diagnosed with diabetes, we suggest that the ORIGIN patients were more similar to our pre-diabetes

patients. Therefore, the results of the ORIGIN trial do not seem to interfere with our findings.

Another finding from the sub-group analysis in the current study was the significantly increased risk of experiencing a fatal AMI among non-diabetic patients with high intakes of n-3 LCPUFAs. Similar to this proposed negative effect in the non-diabetic group, a randomized controlled trial among male patients with angina pectoris revealed an increased risk of cardiac death among participants advised to eat oily fish or FO capsules [170]. They were unable to explain the reason for this adverse outcome. Similarly, in a canine model of post-MI ischemia, high-dose n-3 PUFA supplementation was associated with incident arrhythmias in dogs not originally vulnerable to ischemia [171].

When studying the total patient cohort (**Paper 1**), no clear effects were revealed related to dietary intake of n-3 LCPUFAs. Notably, opposing effects were observed in sub-groups of this cohort (**Paper 2**). Although the number of events was limited within the sub-groups, the suggested associations highlights the importance of acknowledging potential modifying factors which can possibly be hidden in heterogeneous cohorts. Analyses based on sub-phenotypes may thus be important for future personalized nutrition.

5.2 Suggested mechanisms

When relating the rodent results to humans one has to consider the differences between the species, including the extent of PPARα expression which is lower among humans than demonstrated in rat liver. Also, there are major differences in lipoprotein metabolism between rats and humans [23]. However, the rodent model provided us with interesting clues as a basis to explain our findings at a mechanistic level, especially in terms of PPAR mediated effects by FAs. TTA has been shown to

resemble the constituents of fish oil, EPA and DHA, by exerting PPAR mediated effects on mitochondrial metabolism [172].

5.2.1 Changes in fatty acid metabolism

Results from our rodent study (**Paper 3**) indicated an induced mitochondrial and peroxisomal cardiac β-oxidation following TTA treatment, in accordance with findings from two previous studies where an increased myocardial FA oxidation was associated with reduced cardiac efficiency [28,67]. This reduced efficiency has been suggested to be caused by an uncoupling of glucose metabolism, with subsequent acidosis and ischemia [173]. Based on this increase in FA oxidation, TTA has been suggested to have a direct effect on cardiac transcription [67], which is most likely mediated through PPARα activation, since no effect has been observed in PPARα-null mice [28]. Findings also suggested an increased lipogenesis and phospholipid esterification, based on increased cardiac activities of FAS [174] as observed after TTA treatment and of GPAT [175] after both TTA and FO treatment. Being poorly oxidizable FA substrates compared to SFA [176], LCPUFAs in the form of activated acyl-CoAs will most likely be diverted towards lipid synthesis in the liver [175]. Furthermore, LCPUFAs are relatively poor substrates for TG synthesis and are thus mainly incorporated into tissue phospholipids [177]. This could explain why n-3 PUFA levels increase in heart and decrease in liver and plasma following TTA treatment. A previous study did show that TTA induced an overall increased hepatic oxidation of EPA and DHA, concluding that EPA mainly underwent mitochondrial oxidation while DHA oxidation was exclusively peroxisomal [139].

PPARα activation is known to induce degradation of malonyl-CoA [178], a natural inhibitor of CPT-I [179]. However, we observed a decreased cardiac CPT-I activity after TTA administration, which have also previously been shown in liver [180]. Certain conditions can lead towards a reduced FA oxidation efficiency, which might be the case in TTA treated animals even though oxidation capacity in itself seems upregulated. One possible explanation could be a change in carnitine balance between

the tissues and blood. Recent studies in rodents and cell culture show that insulin-resistance leads to increased levels of acylcarnitine intermediates in serum and muscle, due to incomplete β-oxidation [181,182,183]. When tissues have decreased amounts of carnitine, transport of long-chain FAs across the mitochondrial membranes will be impaired while shorter FAs that do not depend on CPT-I will be completely oxidized in the mitochondria [184].

5.2.2 Diet-induced effects on fatty acid composition

The observed FO induced decrease in overall cardiac n-6 PUFA in the rodent study (**Paper 3**) could be explained by dietary n-3 LCPUFAs directing FA metabolism towards the n-3 pathway, due to competing enzymes in the n-6 and n-3 PUFA pathways [77]. This FO effect was different from that of TTA, where ARA was the only cardiac n-6 PUFA being significantly decreased. Still, both TTA and FO treatment induced an increase in cardiac EPA and DHA. In a previous study the PPARα agonist WY-14643 induced a replacement of n-3 PUFA with n-6 PUFA in myocardial phospholipids of isolated cardiomyocytes [178], and thus showed a somewhat different effect from both TTA and FO in the rodent model. The TTA effect on n-3 LCPUFAs was particularly pronounced with an increase in cardiac DPA. In contrast, cardiac DPA was decreased after FO treatment. DPA is an elongation product of EPA, which constitutes only a fraction of total n-3 LCPUFA intake when based on fish or FO intakes [185,186]. Seal oil and meat are natural sources more enriched in DPA [186,187,188]. Effects on the LOX pathway has been demonstrated in platelets were ARA was shunted into the pathway in the presence of DPA [189]. Thus, an apparent link exists between ARA and DPA, which might explain part of the cardiac-specific mechanisms underlying decreased ARA and increased DPA as exerted by TTA. Importantly, DPA is not found to be an active ligand for PPARα [190], and will thus not in itself exert PPAR effects.

In addition to increased cardiac n-3 LCPUFAs, decreased cardiac ARA and decreased hepatic n-3 LCPUFAs, TTA treatment was also associated with elevated levels of

MA both in heart and liver, which could be related to an essential FA deficiency [96]. When dietary levels of the essential FAs LA and ALA are depleted, membrane function and eicosanoid production is maintained through alternative routes due to a metabolic switch that induces the conversion of MA from oleic acid (18:1n-9) [191,192]. Similar findings have previously been reported in animals on a partially hydrogenated FO diet [193] or after treatment with fenofibrate [194], and may be caused by excess PPAR activation. The extensive increase in hepatic FA oxidation as observed after TTA treatment [96] may theoretically lead to a reduced VLDL secretion from the liver. A consequence of this could be that easily oxidizable substrates for cardiac FA oxidation are less available.

Altogether, when comparing the effects on FA composition in heart and liver as exerted by TTA and/or FO in **Paper 3**, there were organ specific changes as previously mentioned, but also opposite effects of the two diets were apparent. In liver, levels of C16:0, total MUFAs, oleic acid, and MA were increased by TTA but decreased by FO treatment. Hepatic levels of n-3 LCPUFAs were reduced after TTA and increased after FO treatment. In cardiac tissue, levels of MA and DPA were increased by TTA but decreased by FO treatment. Similar effects were the increase in cardiac n-3 LCPUFAs and decrease in n-6 PUFAs as exerted by both treatments. Notably, studying the FA composition of the TTA and FO combination group, nearly all cardiac PUFA parameters pointed in the same direction, some more pronounced, as was seen for FO alone. This suggests that possible negative effects, like for instance an increase in MA after TTA administration, were diminished using the combination supplements. Even though the TTA concentrations in the food were equal for both the TTA group and the combination group, the measured levels in the heart differed between the pure TTA group and the combination group. This difference could be explained by either a decreased uptake through the intestine, a competitive mechanism during uptake into the heart muscle, or an increased clearance through the system. We also measured a higher amount of TTA in the heart tissue compared to liver, suggestive of an organ-specific accumulation of the non-oxidizable TTA in the heart.

An increased risk of AMI has previously been associated with increased serum levels of palmitic acid (16:0) and decreased levels of PUFAs, in particular LA [195,196]. In our patient cohort (**Paper 2**), we demonstrated increased serum levels of SFAs and decreased levels of n-6 PUFAs in patients with diabetes, compared to the other patients in the cohort. There was, however, no difference in saturated and *trans* fat intake between the sub-groups. In addition, diabetes patients had a borderline significantly higher intake of total PUFAs. There was no difference in serum n-3 LCPUFA levels between the sub-groups in the cohort, but intakes tended to be higher among patients with diabetes. Diabetes is associated with high levels of non-esterified FAs, which are primarily used in FA oxidation [71] or for TG synthesis [21]. Furthermore, an excess dietary intake of carbohydrates increases *de novo* lipogenesis, which over time may develop into dyslipidemia with a shifted balance in the circulating lipoprotein particles [197]. Based on this, the observed changes in serum levels of SFAs and PUFAs that were not associated to the dietary intake, demonstrate that serum FA composition mainly reflected a dysregulated metabolism in patients with diabetes [198], in line with a probably overall reduced PPAR activity.

5.2.3 Mechanisms in relation to dietary omega-3 effects in humans

Our overall findings did not reveal a clear association between dietary n-3 LCPUFAs and outcome in patients with CAD, but indicated a beneficial effect among patients with diabetes, in line with relations previously seen in patients with reduced ventricular function and heart failure [93]. On the contrary, there seemed to be a disadvantageous effect in patients without diabetes and HbA1c <5.7% who had high intakes of n-3 LCPUFAs. The PPAR effects demonstrated using the animal model, including differences in n-3 LCPUFA levels in heart vs. liver, might help to explain the underlying mechanisms of the proposed conditional favorable/unfavorable effects of n-3 LCPUFAs. TTA treatment has been associated with reduced cardiac efficiency in normal but not in diabetic mice [28,67] and may be related to the observed possible essential FA deficiency in the current study. This may in some ways be in parallel to

the proposed negative effects observed in patients without diabetes and the beneficial effects observed in those with diabetes in relation to intake of n-3 LCPUFA. Although circulating levels of n-3 LCPUFAs are usually directly associated with the dietary intake [18], our results indicate that serum- and tissue levels probably depends to a large extent on *in vivo* metabolism during disease or metabolic changes induced by bioactive FAs. In line with our findings, which we propose to be mainly related to PPAR effects, a recent transcriptomics analysis revealed effects of dietary FAs on cardiac gene expression, suggesting PPARα as an important mediator [199]. Since PPARα activation results in more pronounced effects in rodents compared to humans [26,27], it is difficult to draw direct associations between rodent and human studies. However, mechanisms revealed in animals are very important tools to be able to reach to the next step in human studies when exploring the causal factors of risk associations.

As previously mentioned, PPARs are key regulators of interactions between genes and environmental factors like diet [22]. To demonstrate how human genetics can affect changes in lipid metabolism, a polymorphism in *PPARα* (L162V) has been associated with increased plasma levels of total cholesterol, LDL cholesterol, Apo B, HDL cholesterol, Apo A-I, and apolipoprotein C-III (Apo C-III) [22,200]. There are indications that persons having this polymorphism might have an increased risk of developing some of the components comprising the metabolic syndrome [200]. Furthermore, allelic variability seems to partly explain different individual response on similar diets [22] and a significant interaction between the *PPARα* L162V polymorphism and dietary PUFA intake has been detected, influencing lipid metabolism [201]. Thus, dietary intake of PUFAs and its outcome is probably partly dependent on genetic predisposition [202].

In the current study, the observed risk association between a high n-3 LCPUFA intake and fatal AMI was also related to lower HbA1c levels in the non-diabetic sub-group. One could propose that PPARγ activity might be induced in patients with overall low HbA1c levels, due to its association with improved insulin sensitivity [20]. In relation

to the current findings, a high intake of n-3 LCPUFAs might be unfavourable in subjects having a phenotype with an already high PPARγ activity, proposing a metabolic imbalance between the PPAR subtypes. Such imbalance would be expected to be particularly high in patients with low PPARα activity.

On the other hand, PPARγ activity would be expected to be low in patients with insulin resistance and diabetes. Furthermore, hypertriglyceridemia are generally associated with diabetes [203], and based on the TG lowering effect of PPARα activation, high levels of serum TGs might be a response to reduced hepatic PPARα activity [54]. Diabetes has, additionally, been linked to mitochondrial dysfunction [204], which might also be related to impaired PPAR activity. PPARα is the main driver of mitochondrial β-oxidation [205]. Thus, because n-3 LCPUFAs are potential dual PPARα / PPARγ agonists [206], one can suggest that the beneficial effect in diabetic patients may be particularly pronounced in patients with overall low PPAR activity.

Dienoyl-CoA reductase (*Decr*) null mutant mice are unable to catabolize PUFAs during mitochondrial β-oxidation, leading to an accumulation in the tissues. During fasting, the expression of gluconeogenic genes is inhibited in these animals, leading to hypoglycemia [207]. In fasted PPARα -/- mice there is also a connection between hypoglycemia and accumulation of TGs in both liver and heart tissue [208]. Thus, persons with idiopathic hypoglycemia could hypothetically have a defect PUFA catabolism, making it disadvantageous to increase PUFA intake. The increased risk of fatal AMI which was associated with a high n-3 LCPUFA intake and a lower HbA1c in patients without diabetes might thus also be related to the above mentioned mechanisms. Taken together, these findings suggest that there is a reverse relationship between PUFAs and blood glucose in fasted individuals.

Altogether, further studies are needed to elucidate the complex mechanisms behind the observed associations. However, concerning the low number of events within the sub-groups, together with the observational study design, the data should be interpreted with caution. The proposed beneficial effect among diabetes patients

should preferably be validated in randomized controlled trials. The associations shown in the non-diabetic sub-group, seen together with the uncertain outcomes in previous studies, argue against the use of high-dose n-3 LCPUFA in secondary prevention of CAD among patients without diabetes. Due to the ethical considerations by performing randomized clinical trials investigating proposed negative effects, results in this sub-group of patients with HbA1c levels <5.7% should primarily be verified by other observational studies, by re-analyses of prior randomized studies, as well as by mechanistic studies. The clinical benefits of n-3 LCPUFAs in secondary prevention of CAD are continuously debated. If the current results suggesting different effects in sub-groups of heterogeneous populations are validated by future studies, it might be time for a reassessment of the current dietary advices on n-3 LCPUFA supplementation [8]. Based on our results, and in light of the opposing results and conclusions from other studies, there is still no recipe on an optimal diet for patients with CAD as a whole. The dietary advices of the future should probably be given to each individual based on genetics and disease status and considered in the context of relevant biomarkers and phenotypes.

6. Conclusions

In the total cohort of patients with well-treated CAD and with a relatively high dietary intake of n-3 LCPUFAs, we observed no significant association between intake of n-3 LCPUFAs or fish and risk of coronary events or mortality. After sub-group analysis, a high intake of n-3 LCPUFAs was associated with a significantly reduced risk of AMI in participants with diabetes, but with an increased risk of fatal AMI among non-diabetic participants with HbA1c levels <5.7%.

In rats, long-term administration of the pan-PPAR agonist TTA or high-dose FO were associated with distinct effects on lipid metabolism in the heart, different from liver. FA composition was changed, including an increase in n-3 LCPUFA in rat heart muscle. Furthermore, cardiac mitochondrial and peroxisomal FA oxidation was affected as demonstrated through changes in key enzyme activities and gene expression.

7. Future perspectives

In general, I believe that good health is achieved by consuming a balanced diet in portions adjusted to each individual needs. If one adds regular exercise on top of this, most of the work is done, and the need for supplements is limited. With the increased focus on dietary intake of n-3 LCPUFA nowadays, one must bear in mind that balance should remain as a keyword in a good and healthy diet.

The results presented in this thesis bring out the importance of taking every factor into account before general advice and recommendations regarding the use of dietary supplements are given. Our results demonstrate that different phenotypes may require individual treatment and follow-up. Current work in our group also reveals strong genetic relations to diet and lifestyle. Large-scale studies targeted against the appropriate populations are needed and new biological markers should be established.

Being the cardiac primary transcription regulator, PPARα is an essential FA oxidation determinant in the heart [28]. A reduced cardiac PPAR activation due to impaired lipolysis of TGs could lead to an accumulation of neutral lipids and thus affect mitochondrial function [37]. Based on this, biomarkers for the proposed PPAR imbalance should be established. The acylcarnitines and their metabolites, which hold possible pathophysiological mechanisms related to impaired mitochondrial function, are among the markers we will have further focus on in terms of AMI and mortality. Impaired mitochondrial function has been demonstrated in patients with obesity-related diseases [209], and there has recently been a special attention to dysregulation of FA oxidation. In this context, recent studies have aimed towards the use of acylcarnitine profiling as a new approach to evaluate chronic metabolic conditions [210,211,212]. Elevated fasting values of short-, medium-, and long carbon-chain acylcarnitines have been observed in obese or type 2 diabetic individuals [211], and recent studies in rodents and cell culture showed that insulin resistance lead to

increased levels of acylcarnitine intermediates in serum and muscle, due to incomplete β-oxidation [181,182,183].

The indicated association between low HbA1c, a high intake of n-3 LCPUFAs, and increased risk of fatal AMI among non-diabetics in connection with **Paper 2**, paves the way for investigating this association in more depth [213]. This risk association might be related to the adverse effects of hypoglycemia. In support of this, a previous community-based prospective cohort study demonstrated a significantly increased risk of all-cause mortality in non-diabetic individuals with HbA1c <5.0% [213]. A link has been demonstrated between hypoglycemia, endothelial dysfunction, and increased oxidative stress [214], which could produce a certain metabolic profile. Accordingly, high-dose FO supplementation has also been associated with an increased oxidative damage in rats [96]. Since HbA1c and endpoint data is available on a more extended population than the one used for dietary data, this will be subject for a separate study.

Furthermore, mechanisms underlying excess PPAR activation should be explored into more depth, since this might provide an explanation why n-3 LCPUFA supplements seem to give favorable effects only under certain conditions. It is also notable that PPARα and its agonists hold important properties beyond FA and glucose metabolism, like effects on amino acid metabolism [215,216], which makes it important to do more extensive investigations of PPAR stimulation and cardiac metabolism. Main focus will be on PPAR related markers in response to various dietary fats. To elucidate the specific mechanisms in humans, further studies in human cells and in animals that are more physiological similar to humans are probably necessary. PPARα activation is observed during fasting, activated by adipose-derived FAs, and has been shown to play an important role in carnitine metabolism in mice [217,218], with an increased hepatic carnitine synthesis after PPARα activation [219,220]. However, no such association has yet been described in humans. One group has demonstrated an increased carnitine synthesis and uptake as a result of fasting in pigs [221]. Since pigs are in general suitable models for humans, it is not unlikely that humans could have a similar response upon PPARα activation.

The ultimate aim will be to reveal biomarkers that can be utilized to identify and outline person specific dietary recommendations.

Source of data

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