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Anti-inflammatory, antioxidative and anti-atherogenic bioactivity in marine sources ascribed to nonlipid constituents

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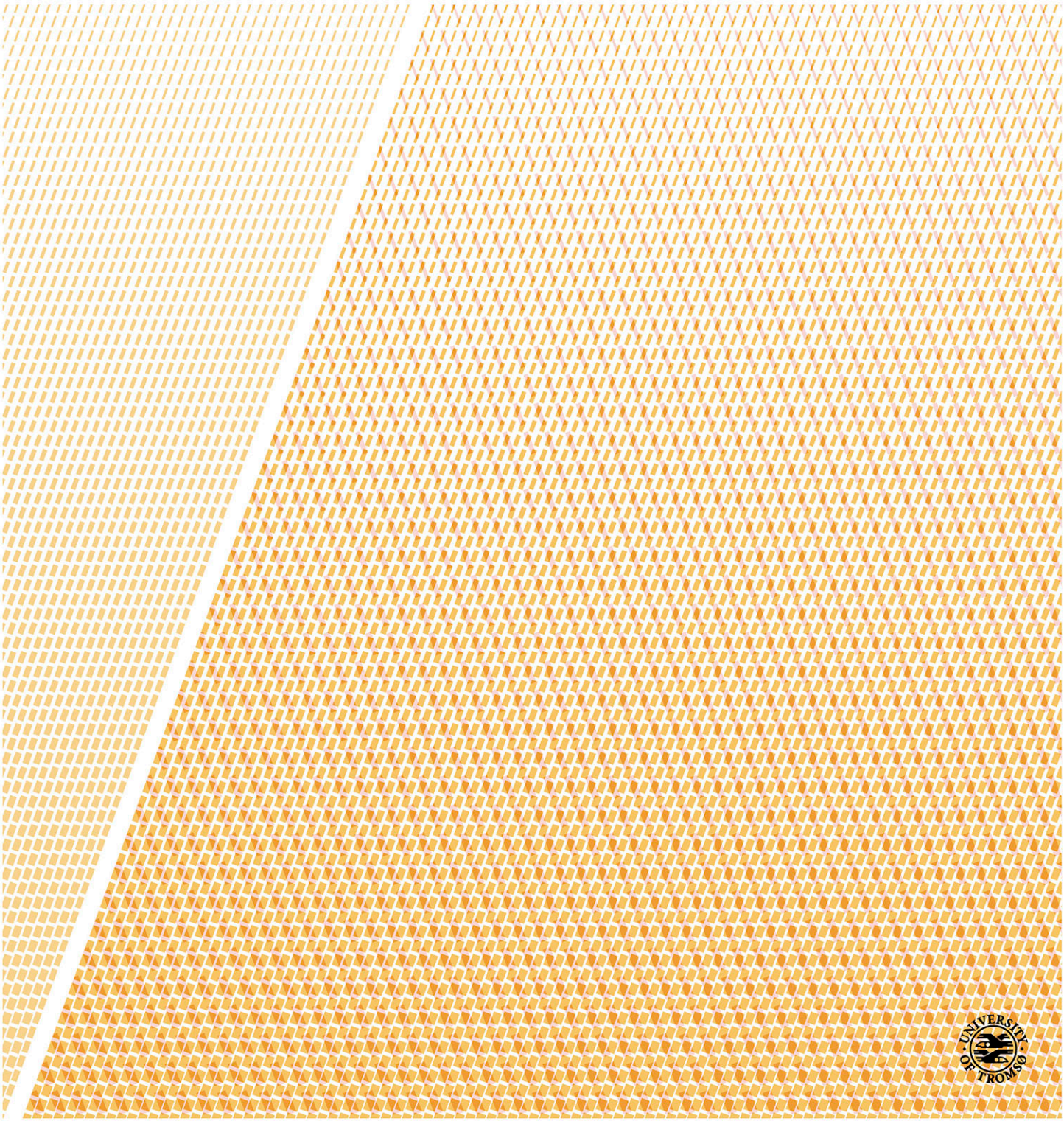

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SUMMARY

Cardiovascular disease (CVD) has been, and continues to be, one of the main causes of global deaths. For decades, fish consumption has been acknowledged to reduce the risk of CVD, and especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to have anti-inflammatory properties. Still, there are indications that beneficial effects are not limited to fatty acids alone. The overall aim of this thesis was to investigate bioactivity in marine sources ascribed to nonlipid constituents.

Lipid-free extracts from cold-pressed whale oil (CWO) and cod-liver oil (CLO) demonstrated high antioxidative capacity in *in vitro* biochemical assays. Furthermore, extracts from CWO had anti-inflammatory activity and reduced Tumour necrosis factor alpha (TNF-α) and Monocyte chemotactic protein 1 (MCP-1) secretion from stimulated THP-1 cells. CLO on the other hand did not display any anti-inflammatory activity in the *in vitro* cell assay.

To evaluate the impact of CWO on atherosclerosis a high fat Western-type diet supplemented with 1% CWO were fed to female apolipoprotein E-deficient mice ($ApoE^{-/-}$) for 13 weeks. The CWO-fed mice had reduced atherosclerotic lesions in the aortic arch compared to control-fed mice. CWO-fed mice also had reduced levels of cholesterol parameters and reduced weight whereas the total antioxidant status and expression of several hepatic genes were heightened compared to control-fed mice.

To evaluate the anti-atherogenic effect from lean protein sources a high fat Western-type diet with the protein replaced with cod-scallop or chicken and fed to female $ApoE^{-/-}$ mice for 13 weeks. This resulted in the reduction of the total aorta plaque burden in cod-scallop-fed mice compared to the total aorta plaque burden in chicken-fed mice. In addition, cod-scallop-fed mice also gained less weight and had lower levels of leptin and glucose when compared to chicken-fed mice.

SAMMENDRAG

Hjerte- og karsykdommer er fremdeles en av de hyppigste dødsårsakene verden over. Gjennom de siste tiårene har det vært velkjent at inntak av fisk reduserer risikoen for hjerte- og karsykdommer, og spesielt fettsyrene eikosapentaensyre (EPA) og dokosaheksaensyre (DHA) er kjente for å ha anti-inflammatoriske egenskaper. Likevel er det indikasjoner på at de fordelaktige effektene ikke er begrenset til bare fettsyrene. Hensikten med denne doktorgraden var å undersøke bioaktivitet i marine kilder som ikke kun kommer fra marine fettsyrer.

Fettfrie ekstrakter fra kald-presset hvalolje (CWO) og tran hadde høy antioksidativ kapasitet i biokjemiske analyser *in vitro*. Ekstrakter fra CWO hadde anti-inflammatorisk aktivitet og reduserte sekresjonen av Tumor nekrose faktor alfa (TNF-α) og Monocytt kjemotaktisk protein 1 (MCP-1) i stimulerte THP-1 celler. Tran på den andre siden hadde ikke noe anti-inflammatorisk aktivitet i denne *in vitro* celleanalysen.

Hun-mus med genetisk apolipoprotein E-mangel ($ApoE^{-/-}$) ble foret med en fettrik diett med 1% CWO i 13 uker for å evaluere påvirkningen av CWO på aterosklerose. Musene som ble foret med CWO hadde mindre lesjoner i aortabuen sammenlignet med musene som ble kontrollforet. I tillegg hadde musene som ble foret med CWO reduserte nivåer av kolesterolparametere og redusert vekt, mens total antioksidant status og utrykket av noen levergener var forhøyet sammenlignet med mus som ble kontrollforet.

For å evaluere anti-aterogen effekt fra magre proteinkilder ble proteinet i en fettrik diett erstattet av torsk-kamskjell eller kylling. $ApoE^{-/-}$ hun-mus ble foret med denne dietten i 13 uker. Den totale plakkdannelsen i aorta var redusert i mus som ble foret med torsk-kamskjell sammenlignet med mus som ble foret med kylling. I tillegg la mus foret med torsk-kamskjell mindre på seg samt hadde lavere nivå av leptin og glukose sammenlignet med mus foret med kylling.

LIST OF PAPERS

Paper I

Mari Johannessen Walquist, Svein Kristian Stormo, Ida-Johanne Jensen, Bjarne Østerud, and Karl-Erik Eilertsen, "Antioxidant and Anti-Inflammatory Activities in Extracts from Minke Whale (*Balaenoptera acutorostrata*) Blubber,"
Mediators of Inflammation (2017), Article ID 3835851, doi:10.1155/2017/3835851

Paper II

Mari Johannessen Walquist, Svein Kristian Stormo, Bjarne Østerud, Edel O. Elvevoll and Karl-Erik Eilertsen "Cold-pressed minke whale oil reduces circulating LDL/VLDL-cholesterol, lipid oxidation and atherogenesis in apolipoprotein E-deficient mice fed a Western-type diet for 13 weeks."
Nutrition & Metabolism (2018) 15:1, doi: 10.1186/s12986-018-0269-8

Paper III

Ida-Johanne Jensen, Mari Walquist, Bjørn Liaset, Edel O. Elvevoll and Karl-Erik Eilertsen. "Dietary intake of cod and scallop reduces atherosclerotic burden in female apolipoprotein E-deficient mice fed a Western-type high fat diet for 13 weeks."
Nutrition & Metabolism (2016) 13:8, doi: 10.1186/s12986-016-0068-z

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ABBREVIATIONS

ABCG5 = ATP binding cassette, sub-family G member 5

ABCG8 = ATP binding cassette, sub-family G member 8

ABTS = 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)

ALA = Alpha-linolenic acid

AOC = anti-oxidative capacity

ApoE$^{-/-}$ = Apolipoprotein E-deficient

ARA = Arachidonic acid

BCA = Brachiocephalic artery

BuOH = Butyl alcohol

CE = Cholesteryl esters

CI = Confidence interval

CLO = Cod liver oil

CO = Corn oil

CVD = Cardiovascular disease

CWO = Cold pressed whale oil

CYP7A1 = Cytochrome P450 7A1

DHA = Docosahexaenoic acid

EPA = Eicosapentaenoic acid

EtOAc = Ethyl acetate

FRAP = Ferric Reducing Antioxidant Power

HDL = High density lipoproteins

HMGCR = 3-hydroxy-3-methyl-glutaryl-Coenzyme A reductase

HPLC = High-performance liquid chromatography

ICAM1 = Intercellular adhesion molecule 1

IFNγ = Interferon gamma

IL-10 = Interleukin 10

IL-1 = Interleukin 1

IL-1β = Interleukin 1 beta

IL-6 = Interleukin 6

IL-35 = Interleukin 35

LA = Linoleic acid

LC-n3-PUFA = Long chain n3-polyunsaturated fatty acid

LDL = Low density lipoprotein

LPS = Lipopolysaccharide

MCP-1 = Monocyte chemotactic protein 1

MI = Myocardial infarction

NCD = Non-communicable diseases

NEFA = Non-esterified fatty acids

NFE212 = Nuclear factor erythroid 2-related factor

NO = Nitric oxide

NSAIDS = Non-steroidal anti-inflammatory drugs

ORAC = Oxygen radical absorbance capacity

Ox-LDL = Oxidized low density lipoprotein

PON2 = Paraoxonase 2

PPARα = Peroxisome proliferator-activated receptor-α

PPARγ = Peroxisome proliferator-activated receptor-γ

PUFA = Polyunsaturated fatty acid

RANTES = Regulated on Activation, Normal T Cell Expressed and Secreted

ROS = Reactive oxygen species

RWO = Refined whale oil

RWO-I = Refined whale oil + extract-I

RWO-II = Refined whale oil + extract-II

SMC = Smooth muscle cells

SR-B1 = Scavenger receptor class B member 1

TAG = Triacylglycerol

TAS = Total antioxidant status

Taurine = 2-aminoethanesulfonic acid

TLC = Thin-layer chromatography

TNF-α = Tumour necrosis factor alpha

VCAM1 = Vascular adhesion molecule 1

VLDL = Very low density lipoprotein

WHO = World Health Organization

1. INTRODUCTION

Non-communicable diseases (NCD) are responsible for 70% of deaths globally each year, with cardiovascular diseases (CVD) as the main type [1]. A *Global action plan for the prevention and control of NCDs 2013-2020* has been developed by the World Health Organization (WHO) [2]. This action plan focuses on cost-effective interventions such as diet and physical activity combined with drug therapy [2]. Diet has a major impact on the general human health and dietary interventions are considered safe and effective means to improve health.

Seafood is considered a healthy low-calorie dietary source of important vitamins, minerals, proteins and lipids [3]. The marine long-chain n3-polyunsaturated fatty acids (LC-n3-PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have anti-inflammatory effects [4-8]. Good sources of LC-n3-PUFA are oily fish and omega-3 supplements [3], however, blubber from marine mammals, such as whales and seals, also have high amounts of LC-n3-PUFA [9].

Epidemiological studies performed in Greenland during the 1970s implied low incidents of CVD in the Inuit population compared to the Inuit population in Denmark [10, 11]. It is, however, important to emphasise that the CVD prevalence has been claimed to be underestimated in the studies of Bang and Dyerberg [12, 13]. Regardless, the low CVD prevalence has been ascribed the diet of the Greenland Inuit, being composed mainly of meat and blubber with a mammalian marine origin [14]. The Inuit research, among other, formed the foundation for human dietary invention studies performed in the 1990s by Østerud and his colleagues [9, 15]. Participants receiving cold-pressed whale oil (CWO) had improved anti-inflammatory status and improved CVD markers [9]. Equal improvements were also observed when CWO was compared to cod liver oil (CLO), although CWO contained less LC-n3-PUFA than CLO [9]. These observations implicated other mechanisms than only LC-n3-PUFA contributing to the anti-inflammatory effect observed.

Marine sources also provide proteins, peptides, amino acids, and other bioactive compounds, in addition to LC-n3-PUFA [3]. Increasing evidence indicate that these contribute to the beneficial effects associated with seafood [3, 16, 17]. Fish and other seafoods are the major

source of 2-aminoethanesulfonic acid (taurine), an organic compound found in most mammalian tissues [18]. Taurine has several important biological roles including osmoregulation, acid conjugation and as an anti-oxidant [19]. Dietary intake of taurine has been indicated to have anti-atherosclerotic properties in an animal studies [18], and epidemiological studies also suggest that taurine is a contributing factor to lower the risk of developing CVD [20-22].

1.1 PROBLEM OUTLINE

For decades, fish consumption has been acknowledged to reduce the risk of CVD, and especially the LC-n3-PUFA, EPA and DHA are recognised as haveing anti-inflammatory effects. Still, previous studies have shown that the observed beneficial effects may not be limited only to the LC-n3-PUFA. Although LC-n3-PUFA is important, the study of lean seafood and other marine components with little or no LC-n3-PUFA, here by removal of lipids from CWO, will make it possible to establish if there is any anti-inflammatory effect of these marine components without the presence of LC-n3-PUFA. *In vitro* studies are important to investigate CVD parameters, however, it is important to proceed with *in vivo* studies to document the possible mechanisms and health effects. These pre-clinical studies may result in future clinical studies.

1.2 PROJECT AIMS

The overall aim of this thesis was to investigate bioactivity in marine sources ascribed to nonlipid constituents, both *in vitro* and *in vivo*, and to provide insight into anti-inflammatory, antioxidative and anti-atherogenic mechanisms related to the prevention of atherosclerosis.

The specific goals in each paper were limited to the following:

Paper I

- To evaluate the *in vitro* antioxidative and anti-inflammatory effects of lipid free cold-pressed whale oil

Paper II

- To evaluate the antioxidative and anti-inflammatory influence of cold-pressed whale oil in an animal (mouse) atherosclerosis model

Paper III

- To evaluate the impact of different protein sources in an animal (mouse) atherosclerosis model

1.3 RESEARCH DESIGN

The schematic overview of the research design for **paper I-III** is shown in figure 1. CWO and CLO were tested in the *in vitro* assays (paper I). These two, together with corn oil (CO), refined whale oil (RWO), RWO-I and RWO-II are included as different feeds in the *in vivo* study (**paper II**). Finally, cod-scallop and chicken were used as different feeds in the last paper (**paper III**).

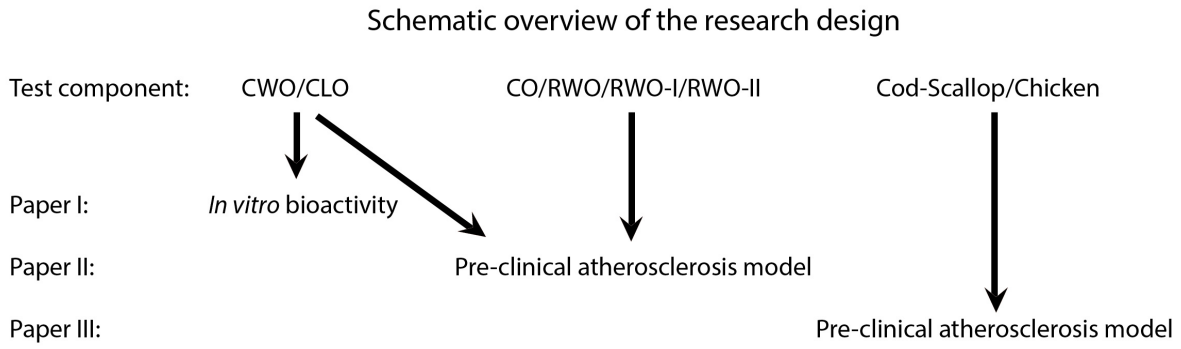


Figure 1 Schematic overview of the research design for paper I-III. CWO = cold-pressed whale oil, CLO = cod liver oil, CO = corn oil, RWO = refined whale oil, RWO-I = refined whale oil + extract I, RWO-II = refined whale oil + extract II.

2 BACKGROUND

2.1 CARDIOVASCULAR DISEASE

The World Health Organization defines CVD as a group of disorders of the heart and blood vessels [23]. Myocardial infarction (MI) and stroke caused by restricted blood flow to the heart or brain, are acute cardiovascular events [23]. Even though CVD mortality decrease in most European countries, CVD still accounts for 45% of all deaths in Europe [24]. Risk factors for CVD are classified as either modifiable or non-modifiable [25]. Non-modifiable risk factors are age, gender and genetics, whereas modifiable risk factors include dyslipidemia, hypertension, type 2 diabetes mellitus, smoking, excessive alcohol consumption, physical inactivity, and obesity [25]. The clinical relevance of the modifiable risk factors is well recognized and the decline in global deaths from CVD are related to change in health behaviour and treatment of these risk factors [26]. Still, the major independent cause of CVD is atherosclerosis, and the main direct cause appears to be rupture of atherosclerotic plaques [27].

2.2 ATHEROSCLEROSIS

Atherosclerosis is an important underlying cause for several types of CVD such as unstable angina, MI and stroke [27]. Atherosclerotic disease may be asymptomatic, or silent, for decades while lipids accumulate and contribute to the formation of lesions in the arterial vessel wall [28]. Atherosclerotic lesions are classified after morphological descriptions [29]. The lesions, or plaque, can cause narrowing of the lumen which may eventually end up in fibrous cap rupture and thrombus formation [30] (figure 2).

The artery wall consists of the three layers tunica intima, tunica media and tunica adventitia (figure 2a). The innermost layer, tunica intima, has residential smooth muscle cells (SMC) and is lined with a monolayer of endothelial cells (figure 2a). The endothelium is an important barrier between the blood and underlying cells and endothelial cells produces several vasodilators and vasoconstrictors to mediate blood vessel tone [31]. Endothelial dysfunction may be a response to cardiovascular risk factors and a key step in the early development of atherosclerosis [32, 33]. Nitric oxide (NO) is a vasodilator that protects against atherosclerosis and promotes normal endothelial function in blood vessels [34]. Reduced release of NO leads

to increased expression of the cell-surface adhesion molecules intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) which facilitate binding of circulating monocytes [35]. After attachment to the endothelial surface, monocytes migrate into the tunica intima where they differentiate into macrophages [36].

Macrophages engulf lipids, and after uncontrolled uptake of oxidized low density lipoprotein (ox-LDL), or with impaired cholesterol release and excessive cholesterol esterification, they transform into foam cells [37]. Foam cells, dying cells and lipids from dead cells, together with cholesterol crystals, accumulate in the lipid core of the plaque [30]. This leads to more advanced lesions with increased macrophage apoptosis and necrosis if the apoptotic and necrotic macrophages are ineffectively cleared by efferocytosis [38]. The necrotic plaque cells can release the cytoplasmic content into the core of the plaque contributing to the necrotic core [39]. Damaged endothelial cells produce mediators that recruits SMC from the tunica media into the tunica intima [30]. Here, the newly arrived SMC proliferate, together with residential SMC [30]. These cells produce collagen, elastin and other extracellular matrix molecules that constitute the fibrous cap covering the growing plaque [30]. Necrotic atherosclerotic plaque with a heightened state of inflammation is more prone to thinning of the fibrous cap [40]. In the event of fibrous cap rupture, the thrombogenic core of the plaque and tissue factors are exposed to the circulating blood leading to activation of the coagulation cascade [41]. Activation of the coagulation system lead to thrombus formation, and the thrombus may stay attached to the ruptured surface and narrow the lumen of the artery, or worse, it may travel with the blood flow and occlude a narrow blood vessel in the circulation [30]. Such occlusion may cause MI or stroke [42].

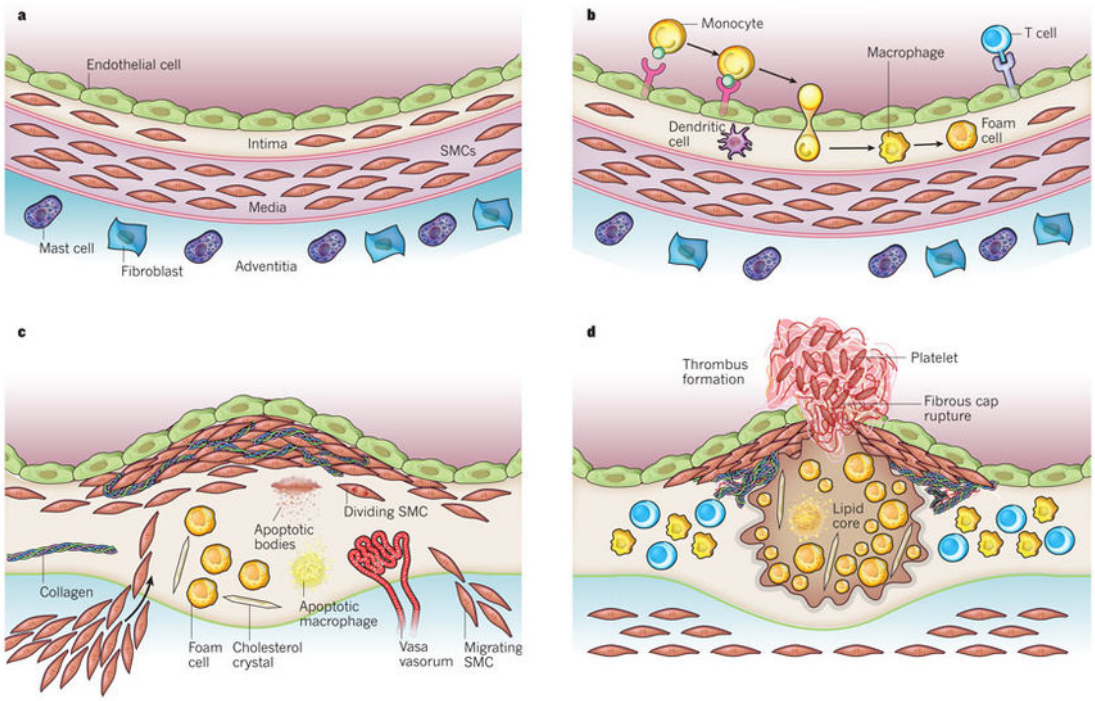


Figure 2 Atherosclerotic process by Libby et al. [30]. The process of atherosclerosis, from normal artery to thrombosis. **a)** The normal artery wall consists of three separate layers; the inner layer (tunica intima), middle layer (tunica media) and outer layer (tunica adventitia). Tunica intima is coated by a endothelial cell monolayer of and has resident smooth muscle cells (SMC). In the tunica media layer, the SMC is organized in a complex extracellular matrix, while the tunica adventitia layer contains microvessels, mast cells and nerve endings. **b)** In the initial phase of atherosclerosis, blood leucocytes adhere to the activated endothelial monolayer and migrate into the intima. Inside the vessel wall, leucocytes mature into macrophages and further into foam cells after lipid uptake. **c)** Lesion advancement includes proliferation SMC, both residential and media-derived, and the increased synthesis of collagen, elastin and proteoglycans. Lipids from apoptotic macrophages accumulate in the lipid core of the plaque, and in advanced plaque cholesterol crystals and microvessels are present. **d)** If the fibrous cap of the atherosclerotic plaque is physical disrupted, blood coagulation components in contact with tissue factors may form a thrombosis. The thrombus extends into the vessel lumen and could obstruct blood flow.

2.3 INFLAMMATION

Inflammation is a protective response from the immune system towards tissue injury or infection [43]. Inflammation is a crucial local response which aims to eliminate the initial cause of cell injury, remove necrotic cells and tissue and facilitate tissue repair [43]. Inflammation is normally a controlled and self-limited mechanism, however, occasionally low-grade inflammation can be switched on by tissue malfunction [43]. A low-grade chronic inflammation will contribute to deterioration of illnesses, such as elevated blood pressure or insulin sensitivity, and diseases like atherosclerosis and diabetes mellitus type 2 are characterized by chronic low-grade inflammation [43]. Inflammations are commonly treated effectively with non-steroidal anti-inflammatory drugs (NSAID) and corticosteroids [44]. Still, this treatment may result in severe side effects such as osteoporosis, stroke and impaired wound healing [45], and it is highly interesting to explore alternative methods to treat inflammation [44].

2.3.1 CHOLESTEROL AND CHOLESTEROL METABOLISM

Cholesterol is crucial for mammals as a structural component in the cell membrane and precursor for biosynthesis of several steroid hormones, vitamin D and bile acids [46]. Cholesterol is either obtained through diet or synthesized in the liver, and cholesterol homeostasis is regulated by faecal excretion of bile acids and by intestinal absorption of dietary cholesterol [46]. Cholesterol and triacylglycerol (TAG) are non-polar lipids and must be transported in association with lipoproteins [47]. The central core of the lipoproteins contains cholesterol esters and TAG surrounded by a hydrophilic membrane consisting of phospholipids, free cholesterol and apolipoproteins [47].

Plasma lipoproteins are, based on their size, lipid contents and apolipoproteins composition, divided into the seven groups very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), lipoprotein (LP), chylomicron, chylomicron remnants and high density lipoprotein (HDL) [47]. These are all, except HDL, considered pro-atherogenic [47]. Chylomicrons and VLDL are produced in the liver and transport TAG, whereas LDL and HDL transport cholesterol back and forth peripheral tissue and liver [47]. LDL are derived from triglyceride depleted VLDL and IDL and enriched with cholesteryl ester (CE) [47]. The majority of cholesterol in the circulation is carried by LDL particles in different size and density [47]. The smallest LDL particles have less affinity for the LDL receptor

resulting in a prolonged circulation time [47]. HDL on the contrary, carry CE from the peripheral tissue to the liver and also hold anti-oxidant, anti-inflammatory, anti-thrombotic and anti-apoptotic properties [47]. The bidirectional flux of free cholesterol between cells and HDL is mediated by scavenger receptor class B type 1 (SR-B1) [48, 49]. Liver SR-B1 has high affinity for HDL binding in humans and mice and mediates the selective uptake of CE into the liver [50]. The obligate heterodimer ATP binding cassette, subfamily G member 5 (ABCG5) and ATP binding cassette, subfamily G member 8 (ABCG8) are located in the hepatocytes and convert CE into bile [51].

As mentioned in section 2.2, macrophages engulf ox-LDL particles and develop into foam cells, the pathological hallmark of atherosclerosis. In addition, high levels of serum LDL cholesterol are related to CVD and atherosclerosis, and considerable effort has been put into reducing circulating cholesterol levels in high risk patients [52].

2.3.2 CYTOKINES

Cytokines are key modulators in inflammation and induce both systemic and local responses in the body [53]. Cytokines and chemokines are involved in all stages of atherosclerosis and greatly influence the pathogenesis of the disease [54]. Cytokines and chemokines may be classified based on their characteristics, pro-atherogenic and anti-atherogenic, related to their function in the development of atherosclerotic plaques [55].

Tumour necrosis factor alpha (TNF-α), interleukin 1 (IL-1) and interleukin 6 (IL-6) are pro-atherogenic cytokines secreted from macrophages, lymphocytes, natural killer cells and vascular SMC [55]. TNF-α and IL-1 signalling affect almost all cells involved in atherogenesis through the p38MAPK/NF-κB pathway [55]. IL-6 is signalling through the Janus kinase 1 and elevated IL-6 serum levels are recognized as an independent risk factor for coronary artery disease [55]. Interleukin 10 (IL-10), transforming growth factor beta and interleukin 35 (IL-35) are considered anti-atherogenic [55]. IL-10 act anti-atherogenic through the down-regulation of TNF-α production, the prevention of ICAM-1 expression on activated endothelial cells and several other mechanisms [55]. TNF-α and IL-1 influence the organization of actin and tubulin

cytoskeletons in the endothelial cells leading to changes in their shape and open gaps between adjacent cells and thus increased permeability for LDL [56].

Chemokines are a subgroup of cytokines that are able to attract cells to a desired location [57]. Monocyte chemotactic protein 1 (MCP-1) is an important chemokine involved in the selective recruitment of circulating monocytes, and regulates penetration and infiltration of monocytes into the inflamed artery wall [58]. Chemokines slow down passing monocytes that starts a rolling movement along the endothelial cells before they migrate across the endothelial layer [59].

Impaired vascular homeostasis resulting in a steady recruitment of monocytes to the inflamed site is a central part of atherogenesis [60, 61]. Depending on the cytokine signal together with macrophage colony-stimulating factor, monocytes in the intima may differentiate into macrophages or dendritic cells [60]. TNF-α, interferon gamma (IFN-γ) and several other cytokines are involved in the foam cell formation, e.g. inhibiting key proteins and hereby decrease cholesterol efflux from macrophages [62]. High cholesterol levels are toxic to cells and will eventually lead to apoptosis and/or necrosis [60, 61]. Cytokines are also involved in the regulation of efferocytosis (removal of apoptotic cells) [63, 64] and defective efferocytosis contribute to lipid accumulation in atherosclerotic lesions [60, 61]. Some cytokines, such as IFN-γ, inhibit synthesis of collagen in SMC, and others may stimulate to SMC apoptosis, hence thinning the fibrous cap, which in turn may lead to plaque rupture and thrombosis formation [62].

2.4 LIPIDS, PROTEINS AND AMINO ACIDS

2.4.1 LIPIDS

Linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) are essential FAs for humans and have to be supplied through our diet [65]. They can, through several steps of chain elongation, be converted into PUFA (arachidonic acid (ARA, 20:4n-6) or EPA (20:5n-3) and DHA (22:6n-3), respectively) [65]. The conversion efficiencies for LA and ALA into PUFA is low [66], and direct dietary uptake of EPA and DHA from seafood is thus more effective.

The predominant PUFA in membrane phospholipids in mammalian tissue are ARA, EPA and DHA, and they have numerous cellular functions affecting eicosanoid synthesis together with membrane fluidity and membrane enzyme activity [67]. Eicosanoids are mediators of inflammation, and the major substrate for eicosanoid synthesis is ARA, which will generate several down-stream pro-inflammatory metabolites [68, 69]. However, EPA and DHA are also substrates for eicosanoid synthesis, and eicosanoids produced from EPA or DHA are known to inactivate ARA-derived eicosanoids and are thus regarded anti-inflammatory [67]. The anti-inflammatory effect of EPA and DHA are also related to specialized pro-resolving mediators, such as E- and D-series resolvins, lipoxins, protectins and maresins [70].

2.4.2 PROTEINS AND AMINO ACIDS

Proteins are essential for body cellular mass increment, recovery and a steady-state maintenance [71]. In general, 9 of 20 amino acids are regarded essential and required through diet [71]. The nutritional value of a protein depend on the amount of essential amino acids, amino acid composition, absorption and utilization after digestion, effects after processing, and source [72]. Taurine is an organic acid containing an amino group created in the hepatocytes or acquired through diet [73]. Taurine is abundant in the brain, retina, muscle tissue and other organs in the body [74]. Taurine is assigned several important functions in the central nervous system and in the conjugation of bile acid [74]. Examples of disease associated with taurine deficiency is cardiomyopathy and renal dysfunction [74]. Taurine is present in most meat used for human consumption and are more abundant in seafood when compared to terrestrial meat, with especially high levels in shellfish and molluscs [75].

2.5 MINKE WHALE

The minke whale (*Balaenoptera acutorostrata*), also known as the common minke whale or the North Atlantic minke whale, is the smallest of the baleen whales [76]. According to the International whaling committee, the population is in a healthy state with approximately 90 000 individuals [77], and minke whale is the only cetacean species commercially hunted in Norway [78]. The minke whale is highly adaptable to changes in the ecosystem in the Barents Sea, and will feed on prey available [79]. The minke whale also migrate north during the spring to feed of krill and copepods together with fish and crustaceans [80]. Minke whale prey consumption contributes significantly to the mortality of their central prey species [81]. The minke whale body is surrounded by a thick blubber, a subcutaneous lipid-rich layer of vascularized adipose

tissue, vital for thermal isolation, structural support and buoyancy [82]. During fasting and breeding blubber serves as the main energy source [82]. The LC-n3-PUFA levels in minke whale blubber are lower than the levels found in their prey, indicating a selective distribution of lipids for storage and for membrane lipids [83].

3 SUMMARY OF PAPERS

Paper I *Antioxidant and Anti-Inflammatory Activities in Extracts from Minke Whale (Balaenoptera acutorostrata) Blubber*

The aim of this study was to investigate if lipid-free extracts from cold-pressed whale oil (CWO) had antioxidative and anti-inflammatory activity ascribed to nonlipid constituents *in vitro*. Cod liver oil (CLO) was also tested and both CWO and CLO had high antioxidative capacity in Oxygen Radical Absorbance Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAP) assays. Several CWO extracts displayed anti-inflammatory activity by reducing tumour necrosis factor alpha (TNF-α) and monocyte chemotactic protein 1 (MCP-1) secretion from lipopolysaccharide (LPS) stimulated THP-1 cells. CWO-BuOH had the most pronounced inhibition of TNF-α and MCP-1, with 50% and 85%, respectively. The extract maintained the inhibitory effect of MCP-1 after long-term storage whereas the TNF-α inhibition was not significant preserved. None of the extracts from CLO displayed any inhibitory effect on the secretion of TNF-α or MCP-1. In conclusion, CWO extracts displayed antioxidative and anti-inflammatory effects without the presence of marine lipids.

Paper II *Cold-pressed minke whale oil reduces circulating LDL-cholesterol, lipid oxidation and atherogenesis in apolipoprotein E-deficient mice fed a Western-type diet for 13 weeks*

The study was design to evaluate the anti-atherogenic effect of cold-pressed whale (CWO) oil in female apolipoprotein E-deficient mice ($ApoE^{-/-}$). Six groups (n=12) of mice were fed a high fat Western-type diet supplemented with 1% CWO, cod liver oil (CLO), refined whale oil (RWO), RWO-1, RWO-II or corn oil (CO). After 13 weeks the mice were euthanized by carbon dioxide inhalation before the organs were harvested and the aorta dissected. CWO-fed mice had reduced atherosclerotic lesions in the aortic arch compared to the CO-fed mice. The levels of LDL/VLDL-cholesterol and ox-LDL-cholesterol were reduced whereas total antioxidant levels status was heightened in CWO-fed mice compared to CO-fed mice. In addition, mice fed CWO gained less weight and several hepatic genes involved in the cholesterol metabolism were upregulated compared to CO-fed mice. Cold-pressed whale oil had beneficial effects on the atherogenesis in $ApoE^{-/-}$ mice with reduced formation of lesions in the aortic arch, reduced cholesterol parameters and reduced weight whereas the total antioxidant status and expression of several hepatic genes were heightened.

Paper III *Dietary intake of cod and scallop reduces atherosclerotic burden in female apolipoprotein E-deficient mice fed a Western-type high fat diet for 13 weeks*

The study was design to evaluate the anti-atherogenic effect of different lean protein sources in female apolipoprotein E-deficient ($ApoE^{-/-}$) mice. Over a timespan of 13 weeks two groups (n=12) of mice were fed a high fat Western-type diet containing cod-scallop or chicken as the protein source. At the end of the study the mice were euthanized by carbon dioxide inhalation before the organs were harvested and the aorta dissected. Compared to the chicken-fed group, the total aorta atherosclerotic plaque burden was reduced with 24% and the thoracic and abdominal parts of the descending aorta were reduced with 46% and 56% in the cod-scallop-fed group. In addition, cod-scallop-fed mice gained less weight and had lower levels of leptin and glucose compared to chicken-fed mice. Two hepatic genes, *Paraoxonase* 2 (*Pon2)* and *Vascular adhesion molecule 1* (*Vcam1)*, were downregulated in the cod-scallop-fed mice compared to chicken-fed mice. Downregulation of *Pon2* suggest lower oxidative stress in the cod-scallop-fed mice. The marine protein from cod-scallop had beneficial effect on the atherogenesis due to the reduced total aorta burden, glucose and leptin levels compared to the chicken protein.

4 METHODOLOGICAL CONSIDERATIONS

4.1 RAW MATERIAL

4.1.1 WHALE OIL

The raw material used to produce the whale oil studied in this thesis was blubber taken from the ventral groove of common minke whale (figure 3). The blubber was provided by Ellingsen Seafood AS (Skrova, Norway). The blubber was frozen to -20°C on board the vessel before transport to our laboratory. Blubber was obtained from the annual commercial hunt of minke whales in Norway occurring in the spring/early summer. Blubber from different time points could have been compared in this study because the fatty acid composition of whale blubber is known to change dependent on factors such as migration and feeding pattern [83]. However, evaluation of such variation was not within the scope of this thesis.

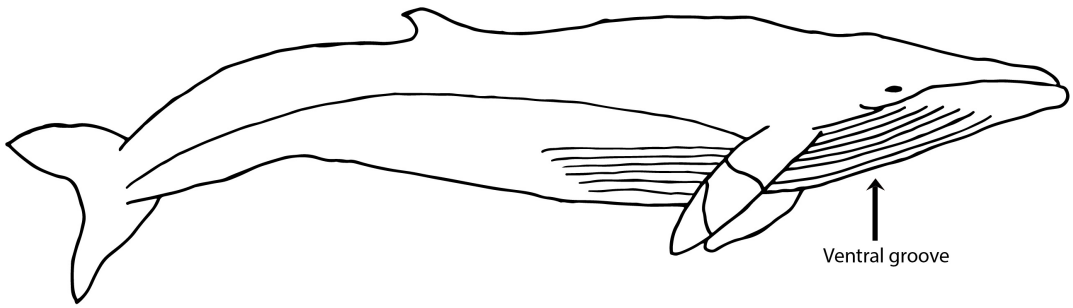


Figure 3 Illustration of the common minke whale and the ventral groove.

4.1.2 COD LIVER OIL AND CORN OIL

The CLO used in **paper I** and **paper II** was commercially available from Orkla Health [84], whereas CO used in **paper II** was bulk oil provided by the diet manufacturer (ssniff Spezialdiäten GmbH).

4.1.3 COD, SCALLOP AND CHICKEN

In **paper III**, the protein sources of the two test diets were chicken and a mixture of wild caught cod and scallop. Cod and scallop were combined to generate a lean diet from a marine protein source high in glycine and taurine (10.1 mg/g and 5.3 mg/g, respectively, **paper III**). Whereas chicken is regarded a healthy terrestrial lean protein source [85] and this diet had less glycine and taurine (5.7 mg/g and 0.1 mg/g, respectively, **paper III**). The wild cod was caught in September in the Northeastern Atlantic while the scallops were commercially available Canadian scallops (*Placopecten magellanicus*). Chicken breasts were bought from Ytterøykylling AS (Ytterøy, Norway).

4.2 EXTRACTION

Today, most of the available dietary marine oils are refined at high temperatures before used for human consumption [86]. The processing steps include bleaching, deodorization and vacuum stripping/distillation and are used to remove unwanted components, e.g. pigments, oxidation products, trace metals, sulphur compounds and contaminants [86]. This type of processing may lead to loss of proteins and trace elements, which means that protective antioxidants also are lost during processing [87]. Antioxidants are added the commercial CLO after refinement to prevent rancidification [84]. In this thesis, the temperature during the extraction of oil from whale blubber was kept below 40°C at all times to protect putative active components in the blubber, hence the oil could be called cold-pressed.

4.3 THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography (TLC) is a planar liquid chromatography with a liquid mobile phase and a silica coated plate as a stationary phase [88]. This method is inexpensive, rapid, simple and widely used for the analysis and isolation of natural products [88]. The samples are spotted on to the plate and solvent migrates up the plate due to capillary forces [88]. The polarity of the

solvents can be adjusted to increase the separation between components in the liquid [88]. A more efficient separation could be achieved using high-performance liquid chromatography (HPLC), which is more precise due to rate control of mobile phase [89]. In this thesis the separation was used merely to classify the lipids and not to quantify or isolate compounds present and TLC was therefore considered the best choice in **paper I**.

4.4 ANTIOXIDATIVE CAPACITY

Antioxidative capacity (AOC) is commonly measured by simplified *in vitro* assessments and several different methods can be used [90]. Since different methods measure factors/parameters differently, the term AOC is rather unspecific and conflicting results between methods are often observed [91].

The assays are usually divided into two groups based on reactions transferring electrons or hydrogen atoms [92]. In this thesis one assay from each group, Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorbance Capacity (ORAC), electron transfer and hydrogen transfer respectively, were used as AOC indicators in **paper I**. FRAP is simple and more rapid than ORAC, but ORAC is performed over a certain time range at physiological pH and 37°C [92]. Therefore, ORAC is considered more physiologically relevant than FRAP. The correlation between ORAC and FRAP is considered low [93]. Both assays have shortcomings. E.g. the ORAC reaction is carried out with an artificial radical in a homogenous system [94] and will therefore not reflect the actual stability *in vivo*. Temperature control throughout the plate is an important issue in ORAC and other temperature sensitive methods. Small differences in temperature between the wells could impact the results. The FRAP assay measure the reducing capacity based on ferric ion (the ability to reduce Fe(III) to FE(II)) [94], and not the antioxidants directly. However, both assays are simple, rapid and inexpensive without the need of special equipment.

Since ORAC and FRAP are not fully comparable and have several shortcomings, measured AOC was used to assess the extracts before further investigations of the anti-inflammatory activity of the extracts. The focus was therefore on the total AOC and not the specific antioxidant present or the mode of action. In this context, technical replicates were considered sufficient and the sample size was not increased to allow statistical analyses of these experiments.

In **paper II**, the AOC was measured with the Total antioxidative status (TAS) kit in serum [95]. The principle of the TAS method is based on inhibition of the absorbance of the radical cation the 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonate (ABTS) [96]. In this method ABTS is oxidized by the peroxidase metmyoglobin (in the presence of H_2O_2). The reduced ABTS molecule is colourless whereas the oxidized cation $ABTS^+$ is blue-green [97]. Antioxidants present in the samples will be quantified based on their ability to suppress oxidation of ABTS and associated colour formation [96]. The method is fast but has several shortcomings, such as the dilution of samples may lead to false-positive results and that antioxidants present may also reduce ferrylmyoglobin radicals instead of ABTS radical, leading to an underestimated antioxidative activity [98]. A decolorization version of TAS method has also been developed, and in that version the ABTS radical is stably formed before the sample is added [99]. However, how well these two version correlate have been a subject of some controversy [99]. When serum AOC was measured with the original TAS assay, the decolorization TAS assay and FRAP assay, the reported AOC levels were highest in the decolorization TAS assay followed by the original TAS assay, while the lowest serum AOC was measured by the FRAP assay [97]. This may be a result of fast-acting antioxidants capable of reducing the ferrylmyoglobin radical, and intermediate radical in the original TAS assay [98], whereas the FRAP assay measure nonprotein AOC and protein are the main antioxidative component in serum [97]. Another study compared original TAS assay with ORAC assay and FRAP assay reporting the AOC order as ORAC > original TAS > FRAP [100]. Importantly, the TAS method was already established and validated in our laboratory for measuring AOC in serum from mice and were thus chosen as the preferred method in this thesis.

4.5 ANIMALS IN LABORATORY EXPERIMENTS

Use of animals for scientific purposes has been a subject for debate for many decades and this is a matter with great ethical concerns. Ethical guidelines, laws and regulations are established to control animal experiments. In 1959 Russel and Burch wrote the book "Principles of Humane Experimental Technique", and introduced the guiding principle of the three R's in animal research [101].

The first R stands for Replacement, which means that research should replace living animals with *in vitro* techniques such as chemical, biochemical and cell culture assays whenever

possible. To rely only on *in vitro* studies will not provide documentation of mechanisms and potential health effects *in vivo*, however, it is important to perform *in vitro* studies before proceeding to *in vivo* studies. In this thesis, **paper I** is an *in vitro* paper using chemical assays and a cell model to test our extracts. The animal studies in **paper II** were based on the results from **paper I** and the previous results from the nineties [9, 15]. Jensen et al performed *in vitro* digestion experiments with cod [102] and studies have been performed on taurine, cod and scallop [103-107], previous to our animal study in **paper III**.

The second R stands for Reduction of the number of animals used in each experiment [101]. This is obtained by power analysis and sample size calculation to avoid using too many individual animals. Studies using standardized animals (e.g. genotype) with little or minimal genetic variation contributes to reduce the animals needed to obtain significant results. There are no *in vitro* models for investigation of atherosclerotic progression, and human studies have several limitations and obvious restrictions. Although several larger animals such as rabbit and pigs have been used to investigate atherosclerosis disease mechanisms, and effects of drugs and diets [108, 109], mice are often considered the species of choice due to their short life span, inexpensive housing and breeding, and easy standardization [110]. Wild mice do not develop atherosclerotic lesions at a high fat modified diet [111], however, it is relatively easy to introduce genetic modifications in mice [110]. In atherosclerosis prone mice, lesions develop over a period of months and due to the small size, the required amount of the drug or compound to be investigated is low [110]. Genetically modified mice models still have several important limitations. For instance, lipoprotein profiles in mice are not identical to humans and mice do not develop unstable plaques that may rupture and lead to thrombosis which is a crucial step in human disease aetiology [110, 112]. Still, the initial phase of atherosclerotic development seems to be similar in mice and humans with the fatty streak developing into advances lesions with a fibrous cap [110, 112]. In this thesis, apolipoprotein E-deficient ($ApoE^{-/-}$) mice were chosen as the model due to their spontaneous development of atherosclerotic lesions. This rapid development of atherosclerosis is a result of the impaired plasma lipoprotein clearing in the $ApoE^{-/-}$ mice [113]. This mouse model is recognized as a suitable model to investigate effects of anti-atherogenic diets [114] and is a mouse model our group has previously experience with [115-117].

The last R stands for Refinement, referring to the effort and methods aiming to minimise pain, distress and suffering that may be experienced by laboratory animals [101]. For instance,

improvement of the housing conditions and protocols used to minimize the animal suffering. UIT – The Arctic University of Norway has a specialized animal facility with trained employees and a veterinarian in charge. The animal facility emphasize enhanced animal welfare and conditions are adapted to meet this. It is also mandatory for every researcher working with animals to have obtained FELASA B/C accreditation before starting the animal studies.

4.6 DETERMINATION OF PLAQUE BURDEN IN AORTA

After carefully dissection of the aorta from the mice carcass, any remaining periadventitial adipose tissue was removed before the aorta was opened longitudinally. Aortas were stained with Oil Red O staining and mounted *en face* on slides under coverslips. Following scanning of slides, image analysis revealed the amounts of atherosclerotic plaques burden. The atherosclerotic development was reported relatively to the total area of each given artery.

Even though *en face* lipid staining of the aortic surface using Oil Red O staining is the established methodology to quantify atherosclerotic plaque burden in mice [118], the method has several drawbacks; The method is time consuming, the aortas need to be dissected from the carcass and cleaned precisely from any periadventitial tissue. Staining will only provide information of the area covered by plaque and not the three-dimensional structure which makes it difficult to determine the developmental stage of the lesions. Furthermore, the aorta will be physically distorted after the treatment and not usable for more detailed morphological analysis.

Nevertheless, the *en face* evaluation is superior to e.g. cross-section determinations [119] due to accurate determination of shape, number and distribution of lesions throughout the entire aorta [120]. There are several other methods also available for quantitative determination of plaque burden in atherosclerosis mice models such as immunohistological staining [121] and MicroCT imaging [122]. Loyd et al. compared MicroCT imaging to *en face* by performing them successively [122]. The authors suggested that further studies should use both methods in combination to develop a further understanding of plaque pathologies. However, MicroCT imaging requires very expensive equipment currently not implemented and available in our laboratory.

Another procedure considered for this thesis was to quantify the lesions in the brachiocephalic artery (BCA or innominate artery). The BCA supplies blood to the right arm (forefoot in the

mouse) and the head and neck and BCA is the first branching artery from the aortic arch. However, the BCA is very small and this method requires meticulous dissection to avoid artefacts associated with strain and tare of the artery.

4.8 SERUM OR PLASMA FOR ANALYSIS

In mouse studies, the obtainable serum or plasma levels from each mouse is an important limiting factor. From repeated sampling, the recommended sampling volume is 10% of the circulating blood volume [123], following this 50-100 µl is the maximum obtainable serum/plasma volume. For the final blood sample collected when the study was terminated, 500 µl is the maximum obtainable volume. Our studies follow the institute's ethical requirements which requires that animals are dead before taking the final blood sample. Another consideration when it comes to analysing proteins, lipids and lipoproteins in circulating blood is whether to use plasma or serum. Plasma has some practical limitations compared to serum due to the need for immediate centrifugation and freezing after sampling to avoid bias from different processing. Previous experiences in our research group showed that heparin-, citrate- and EDTA-plasma frequently contained particles interfering with lipid/cholesterol analysis. Serum samples were therefore chosen in the assays, even though serum is not an optimal sample media for evaluating cytokines, chemokines and other inflammatory markers.

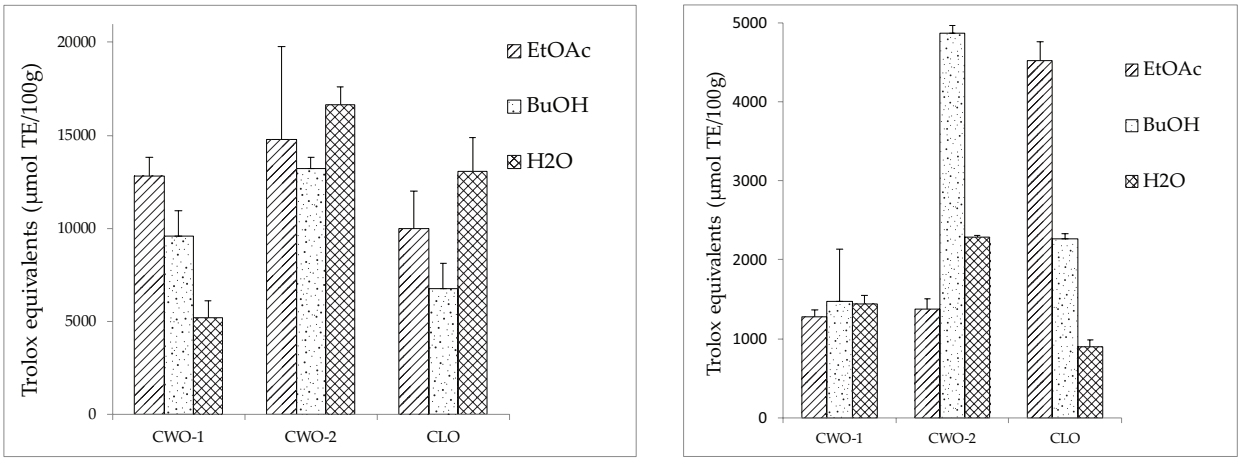
5 DISCUSSION OF MAIN RESULTS

The overall purpose of this thesis was to investigate anti-inflammatory bioactivity, as well as antioxidative and anti-atherosclerotic effects from marine sources with combinations of *in vivo* and *in vitro* experiments to provide some insight in the putative cardio-protective effects. In **paper I**, the lipophilic part of CWO and CLO was removed and the remaining part was investigated for antioxidative and anti-inflammatory effects not related to LC-n3-PUFA. In **paper II**, the anti-atherogenic effects of whale oil in different variants were evaluated after female ApoE$^{-/-}$ mice had been fed high fat Western-type diets supplemented with 1% of the different oils for 13 weeks. **Paper III** evaluated the putative anti-atherogenic effects in female ApoE$^{-/-}$ mice fed a high fat Western-type diet with lean seafood protein source or lean terrestrial protein source.

5.1 ANTIOXIDATIVE CAPACITY

Antioxidant activity has been ascribed many of the observed health effects of bioactive compounds and carotenoids [91]. Two methods commonly used to assess the AOC *in vitro* are FRAP and ORAC [93] and the results provided the fundament for further investigation of CWO. Reactive oxygen species (ROS) are produced and tightly regulated in normal cellular metabolism [124].

Antioxidants are naturally present in the body, however, if there is excessive production of ROS or not enough antioxidants present the body may experience oxidative stress and cellular lipids, proteins and DNA may be harmed [124]. Antioxidants protect oxidisable substances by inhibiting or delaying the oxidation process, by removing oxidative damage or by inhibit ROS production [125-127]. In the *in vitro* assays in **paper I** both CWO and CLO had high AOC (figure 4), however, the dry matter yield showed considerable less total antioxidants in CLO compared to CWO.



(a) (b)

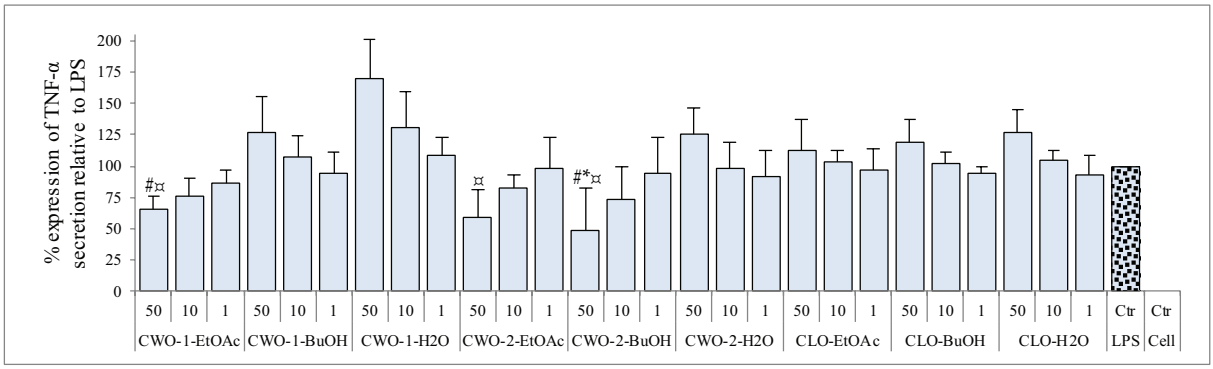
Figure 4 Antioxidative capacity retrieved from paper I. CWO-1, CWO-2 and CLO were extracted sequentially with EtOAc, BuOH and H_2O and the result are presented as Trolox equivalents (µmole TE/100g). (a) Oxygen Radical Absorbance Capacity (ORAC) assay (b) Ferric Reducing Antioxidant Power (FRAP) assay.

5.2 ANTI-INFLAMMATORY EFFECT ON CYTOKINE SECRETION

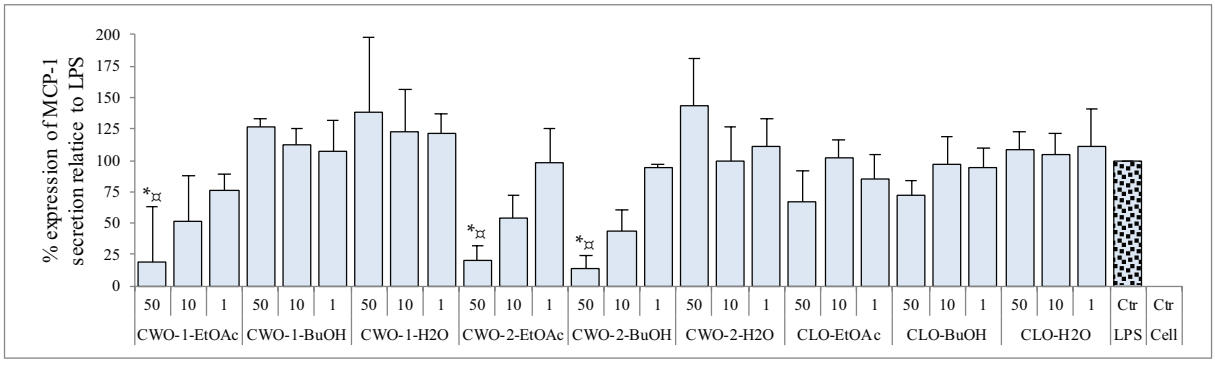
The macrophage like THP-1 cell line were established in 1980 [128] and have since been recognized as a suitable *in vitro* model to examine the regulatory and functional mechanisms of monocytes and macrophages in the cardiovascular system [129]. Anti-inflammatory effects were assessed based on the extracts´ abilities to inhibit lipopolysaccharide (LPS) induced TNF-α and MCP-1 secretion from differentiated THP-1 cells. The CWO extracts inhibited MCP-1 secretion (figure 5b) more pronounced than they inhibited TNF-α secretion (figure 5a). MCP-1 plays a crucial role in atherosclerosis and is involved in the migration and infiltration of monocytes and macrophages in the artery [58]. An MCP-1 inhibitor may be an attractive drug candidate to delay the atherosclerosis progression or other chronic inflammation diseases.

After more than four years of storage in the freezer (at -20°C), the CWO extracts still had an inhibitory effect on LPS-induced MCP-1 whereas inhibition of LPS-induced TNF-α secretion was not significant. The stored extracts did not affect Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) secretion from the LPS-treated THP-1 cells. After LPS binding to toll like receptor 4, different signalling pathways are activated to produce MCP-1 and RANTES. MCP-1 is produced through the MyD88-dependent pathway together with other pro-inflammatory cytokines such as TNF-α [130, 131] while RANTES are produced through the MyD88-independent pathway together with interferons [132, 133]. The extracts derived

from CLO on the other hand, did not inhibit TNF-α nor MCP-1 secretion from the THP-1 cells when compared to the LPS controls. LPS-induced Interleukin 1-beta (IL-1β) secretion was also measured from the THP-1, however, none of the extracts tested inhibited secretion. IL-1β has a different secretion mechanism than TNF-α, MCP-1 and RANTES due to the lack of a secretory sequence to be translocated to ER lumen [134]. This may explain why none of the extracts tested inhibited IL-1β secretion. CLO derived extracts together with CWO derived extracts all had high AOC, but none of the CLO derived extracts displayed any anti-inflammatory activity. This might be explained by the high temperatures used in the refining process of commercial CLO leading to degradation and loss of putative anti-inflammatory compounds.



(a)



(b)

Figure 5 Anti-inflammatory assay retrieved from paper I. ELISA assay displaying relative response of Tumour Necrosis factor alpha (TNF-α) and Monocyte chemoattractant protein-1 (MCP-1) presented as mean SD. (a) Secreted TNF-α relative to control. *$p<0.05$ related to LPS control $^{\#}p<0.05$ related to CLO-BuOH 50 µg/ml $^{¤}p<0.05$ related to CLO-H_2O 50 µg/ml. (b) Secreted MCP-1 relative to control. *$p<0.001$ related to LPS control $^{¤}p<0.001$ related to CLO-H_2O 50 µg/ml.

5.3 EFFECT OF COLD-PRESSED WHALE OIL ON ATHEROSCLEROSIS IN APOLIPOPROTEIN E-DEFICIENT MICE

In **paper II** the effect of CWO on the atherosclerotic progression was evaluated in female ApoE$^{-/-}$ mice fed high fat Western-type diets. The diets were supplemented with 1% CWO, or 1% RWO enriched with two different types of extracts using CO and CLO as control diets.

When compared to CO-fed mice, CWO-fed mice had reduced atherosclerotic plaque burden in the aortic arch, whereas no significant differences were observed in the thoracic aorta, abdominal aorta or total aorta (figure 6). CWO-fed mice also had reduced LDL/VLDL-cholesterol and ox-LDL levels compared to CO-fed mice, and upregulated TAS compared to both CO-fed mice and CLO-fed mice. This is in accordance with **paper I**, demonstrating that extracts from CWO had *in vitro* antioxidative effects with higher total antioxidants than CLO. Similar anti-atherosclerotic effects were observed in a study feeding seal oil mixed with extra virgin olive oil, known to hold protective antioxidants, to ApoE$^{-/-}$ mice [116].

ROS accumulation results in oxidative stress causing lipid peroxidation, oxidative damage and drive atherogenesis whereas antioxidants may contribute to keep ROS production at a normal level, thus delaying atherosclerotic development [135]. CWO has, as described in **paper** I, high AOC, suggesting that CWO do not require further addition of antioxidants. Together, increased TAS levels and reduced ox-LDL levels may explain the observed reduction of atherosclerotic lesions in the CWO-fed mice compared to the CO-fed mice. TAS was unaffected in the CLO-fed mice compared to the CO-fed mice despite the high AOC levels described in **paper I**. This indicate that more than antioxidants contribute to the reduction of atherosclerotic plaque observed in CWO-fed mice.

Cholesterol is vital for mammalian cells, however, an accumulated level of circulating LDL-cholesterol is a major risk factor to develop atherosclerosis or hyperlipidaemia. Several hepatic genes are involved in the feedback-regulated process of the biosynthesis of cholesterol and to investigate the impact of the diets on these genes a predesigned TaqMan gene expression assay with a selection of hepatic genes was performed.

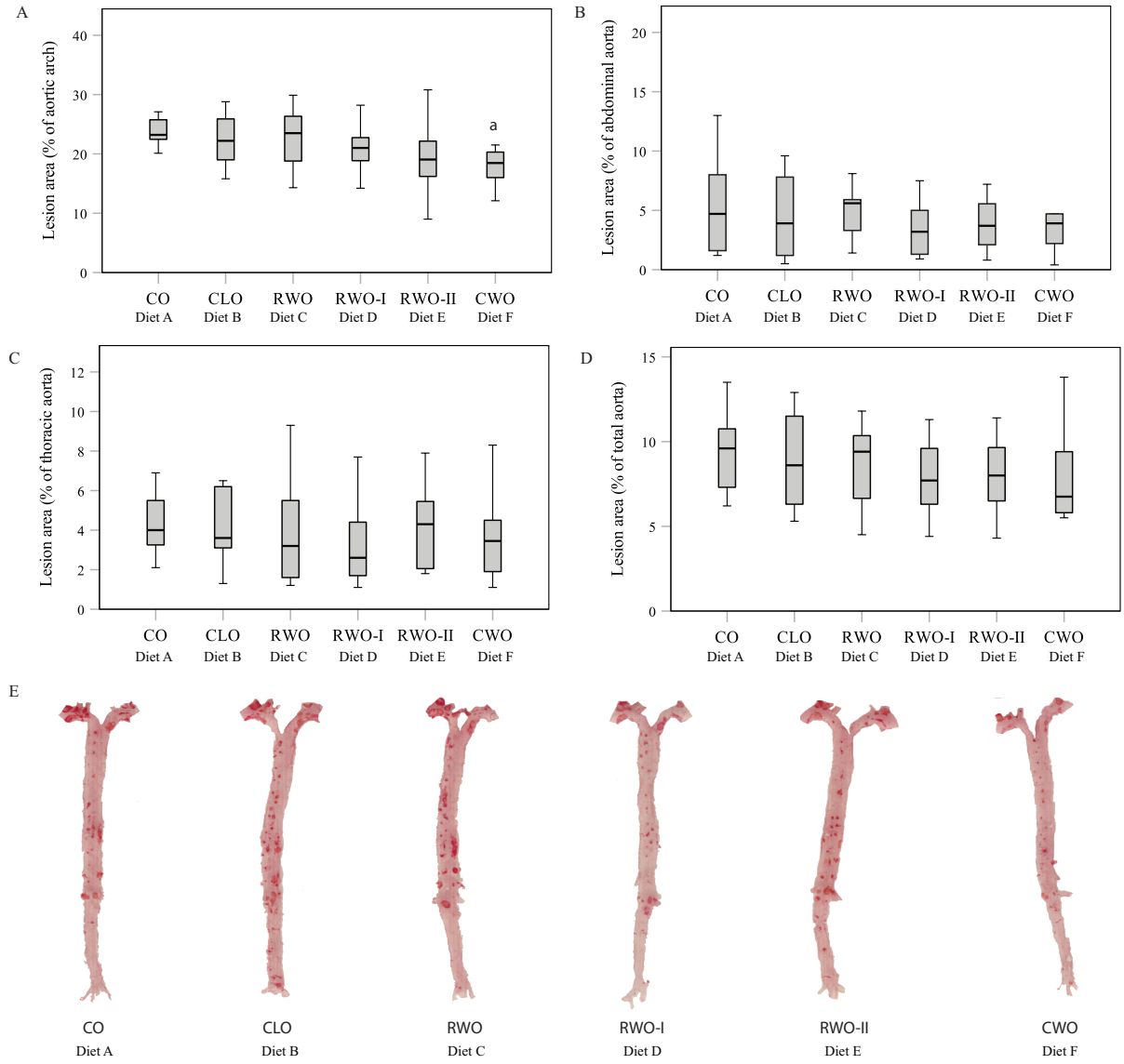


Figure 6 Atherosclerotic plaque burden retrieved from paper II. Female apolipoprotein E-deficient mice were, for 13 weeks, fed a high fat Western-type diets supplemented with different oils. (a-d) Diet A (n=11), diet B (n=9), diet C (n=11), diet D (n=11), diet E (n=12), diet F (n=10). **a** represents significant difference from mice fed diet A (CO) ($p<0.05$). (e) representative aorta from group A-F.

The CWO-fed mice had moderately up-regulated *Abcg5*, *Abcg8* (p=0.064), *sr-b1* and *Peroxisome proliferator-activated receptor α* (*Pparα*). Overexpression of SR-B1 in mice leads to increased faecal cholesterol clearance [50] and hepatic SR-B1 is a positive regulator of macrophage reverse cholesterol transport *in vivo* [136]. The heterodimers ABCG5 and ABCG8 are important contributors to the maintenance of the cholesterol balance by converting CE, or other sterols, into bile for excretion in the large intestine [51] and hence prevent accumulation

of sterols from the diet [137]. Overexpression of *Abcg5* and *Abcg8* increase biliary cholesterol secretion and reduce inflammatory markers in the liver [138] and has, as the *sr-b1* gene product [139], an atheroprotective role in mice. This may explain the reduced LDL-cholesterol and ox-LDL-cholesterol observed in the CWO-fed mice compared to the CO-fed mice. However, none of the inflammatory markers had reduced levels when assessed both by hepatic gene expression analysis and by multiplex ELISA of serum protein levels. Also, the CLO-fed mice had reduced gene expression of *Abcg8* and *sr-b1*, but the LDL/VLDL-cholesterol and ox-LDL levels were not reduced compared to the CO-fed mice. Another gene which may be involved in the observed effects is *Pparα*,which is a nuclear receptor participating in uptake, transport and reduction of fatty acids and TAG synthesis [140]. Natural ligands for PPARα include LC-n3-PUFA [141]. All diets used in this study, except the CO-control, contain marine oils which may activate PPARα and contribute to the beneficial effects observed.

The extract(s) was reconstituted with RWO to investigate whether the anti-inflammatory activity previously observed with the CWO, could be ascribed to the fatty acids (RWO), the water-soluble components in the whale oil (RWO-I), or the water-soluble components in the residual whale blubber (RWO-II). Even though the results were not conclusive, both RWO-I and RWO-II reduced serum LDL/VLDL-cholesterol and ox-LDL concentration whereas the serum TAS was increased compared to the CO-fed mice. From the gene expression studies, the hepatic expression of *Abcg5*, *Abcg8*, *Cytochrome p450 7A1* (*Cyp7al)*, *3-hydroxy-3-methyl-glutaryl-Coenzyme A reductase (Hmgcr)*, *Sr-b1*, *Pparα* and *Peroxisome proliferator-activated receptor γ* (*Pparγ*) were all increased in mice fed RWO-II when compared to the CO-fed mice. This may indicate that at least the RWO-II extract affected the same metabolic processes as CWO, however, these effects were moderate as no significant reduction was observed for the atherosclerotic plaque when compared to the CO-fed mice. The most established effect of LC-n3-PUFA supplementation is reduction of circulating TAG levels, however, the dosage has to be pharmaceutical (3-4 g/day) to achieve optimal TAG lowering [142, 143]. In this thesis, the levels of LC-n3-PUFA were far below pharmaceutical dosage and at a level which is obtainable through normal diet and food supplement. Thus, as anticipated, the CWO-fed mice had unaltered TAG serum levels compared to the CO-fed mice.

5.4 EFFECT OF DIFFERENT PROTEIN SOURCES ON ATHEROSCLEROSIS IN APOLIPOPROTEIN E-DEFICIENT MICE

Paper III evaluated the effect of a marine protein source on atherosclerosis development in female ApoE$^{-/-}$ mice fed high fat Western-type diets. The standard protein source casein was replaced with i) cod fillet and scallop muscle combined 1:1 on nitrogen basis as a marine protein source or ii) chicken breast as a terrestrial protein source. When the composition of the diets was analyzed, the cod-scallop diet had 1.9 g/kg cholesterol whereas the chicken diet had 2.0 g/kg. However, this difference was too low to have an impact on the atherosclerosis progress. When compared to the mice fed the chicken diet, the cod-scallop fed mice had reduced plaque burdens in the aorta thoracic, abdominal and total area (46%, 56% and 24% respectively) (figure 7).

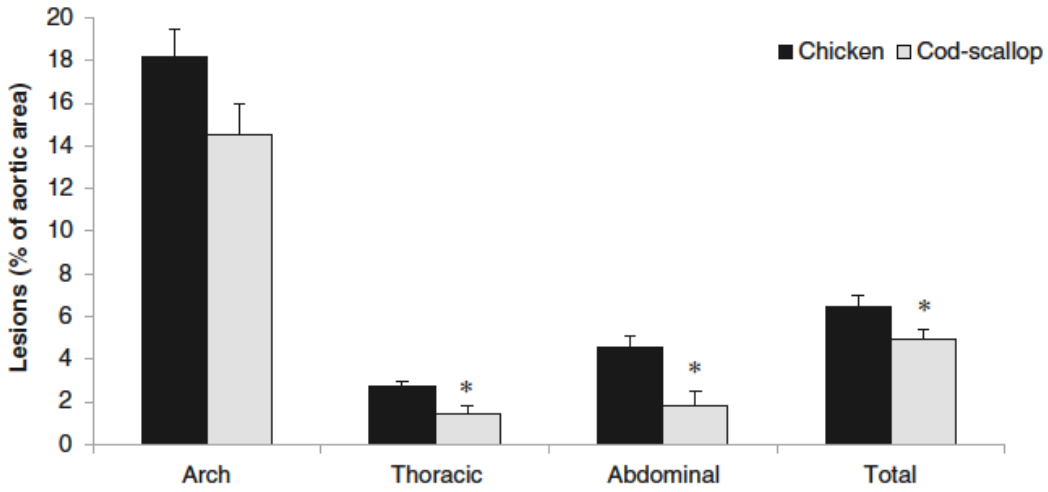


Figure 7 Atherosclerotic plaque burden retrieved from paper III. Female apolipoprotein E-deficient mice were fed high fat Western-type diets for 13 weeks with cod-scallop or chicken as the protein sources. *represent the significant difference between cod-scallop fed mice (n=10) and chicken-fed mice (n=12). Data are presented as mean ± SEM.

A tendency for reduction of the plaque burden in the aortic arch as well as the serum cholesterol levels were observed in mice fed the cod-scallop diet, however, these effects were not significant. The chicken diet had low content of taurine, whereas the cod-scallop diet contained 5.3 mg/g taurine. Taurine has been shown to reduce atherosclerotic lesions in ApoE$^{-/-}$ mice [144], and taurine is known to reduce circulating cholesterol levels by increasing excretion of bile acid [145]. When atherosclerotic burden is reduced without a parallel

reduction of cholesterol levels, another mechanism than lowering of circulating cholesterol levels is needed to explain the effect on plaque burden.

Oxidative stress is an important part of atherosclerosis [146] and endogenous antioxidants such as Paraoxonase 2 (PON2) are upregulated in response to oxidative stress [147]. The chicken fed mice had elevated levels of PON2 compared to the cod-scallop fed mice signalling less oxidative stress in the cod-scallop fed mice which might have led to the observed reduced atherosclerotic lesions. Such beneficial effect may also partly be aascribed to taurine from the cod-scallop diet as taurine is known to have antioxidant activity [103, 148]. The *Vcam1* gene was also down-regulated in cod-scallop fed mice. The expression of the cell-surface adhesion molecule VCAM1 is upregulated in endothelial cells during inflammation and it enhance the binding of circulating monocytes and their migration into the intima in the vessel wall [149]. The down-regulation thus, implicates less inflammation in cod-scallop fed mice compared to chicken fed mice, however, none of the other hepatic inflammation genes studied differed between the two groups.

Even though they had similar feed intake, the cod-scallop fed mice gained less weight compared to the chicken fed mice. The cod-scallop fed mice also had less adipose tissue (by weight), which is the primary production site of leptin. Cod-scallop fed mice consequently had less leptin compared to chicken fed mice. Leptin is a key hormone in the regulation of food intake and energy expenditure balance [150]. Leptin is linked closely to atherosclerosis and may directly influence the growth of atherosclerotic plaque through several mechanisms [151, 152]. In one study, leptin-treated $ApoE^{-/-}$ mice had greater atherosclerotic burden than the vehicle-treated control mice [153], which is in accordance with our findings.

6 CONCLUSIONS

When CWO was deprived of the lipophilic part it still contained antioxidants and had anti-inflammatory activity that could not be ascribed to LC-n3-PUFA activity. In comparison, CLO deprived of the lipophilic part also contained antioxidants but had no anti-inflammatory activity. When CWO was given to atherosclerosis prone mice, a reduced formation of atherosclerotic lesions in the aortic arch compared to CO-fed mice was observed. CWO-fed mice also had reduced body weight and serum levels of LDL/VLDL, ox-LDL, together with elevated serum total antioxidant status. Interestingly, the same effect was to some extent seen in RWO-II-fed mice.

The cod-scallop fed mice displayed favourable metabolic effects, reduced atherosclerotic burden, reduced body weight, reduced visceral body tissue, reduced serum glucose and reduced leptin levels compared to the chicken-fed mice. This thesis adds to the notion that CWO has anti-inflammatory and antioxidative effects and it demonstrates for the first time that CWO prevent the formation of atherosclerosis. Furthermore, this thesis provides novel insight into the putative protective mechanism of dietary supplementation of CWO and cod-scallop in CVD and demonstrates that these beneficial effects are not limited to the well-known LC-n3-PUFA effects.

7 FUTURE PERSPECTIVES

To further elucidate the atheroprotective effects from CWO demonstrated in this thesis, the unknown bioactive compound(s) should be isolated and investigated further with bioprospecting screening assays. A putative isolated compound as a feed supplement in high fat Western-type diet fed ApoE$^{-/-}$ mice may provide more insight into the impact of CWO on atherogenesis. In addition, cod-scallop as a marine protein source should be compared to lean meat, red meat and processed meat for further *in vitro* bioactivity assays. The animal studies may be extended for a longer period of time to give more consistent results and this will most likely give more pronounced group-differences.

In such animal studies of mice intestines can be sampled to isolate RNA and to quantify regulation of genes involved in protein and lipid metabolism. In addition, mice faeces may be collected and analysed in future the animal studies. This will provide insights into true digestion

and excretion mechanisms involved in the cholesterol metabolism. The gut microflora in the mice is another notable aspect to look into. Another interesting approach would be to investigate whether some of these supplements may be potent enough to reverse already established atherosclerosis in mice.

Together, these investigations are likely to increase our knowledge, and understanding of the complex mechanisms involved in both formation and prevention of atherosclerosis and demonstrates the importance of further investigations.

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Paper I

Paper II

Paper III