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

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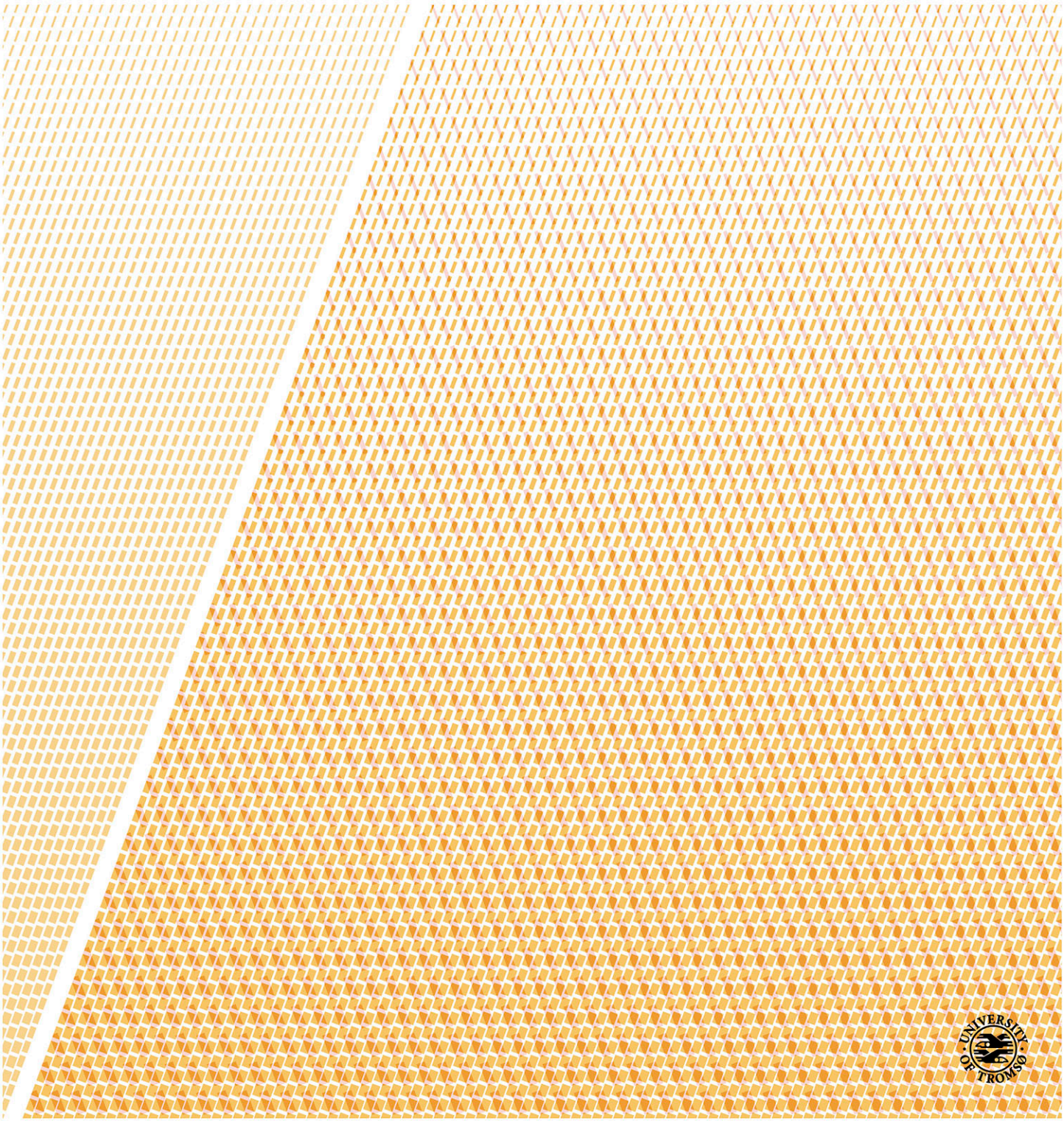

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SUMMARY

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

LIST OF FIGURES

TABLE OF CONTENTS

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**).

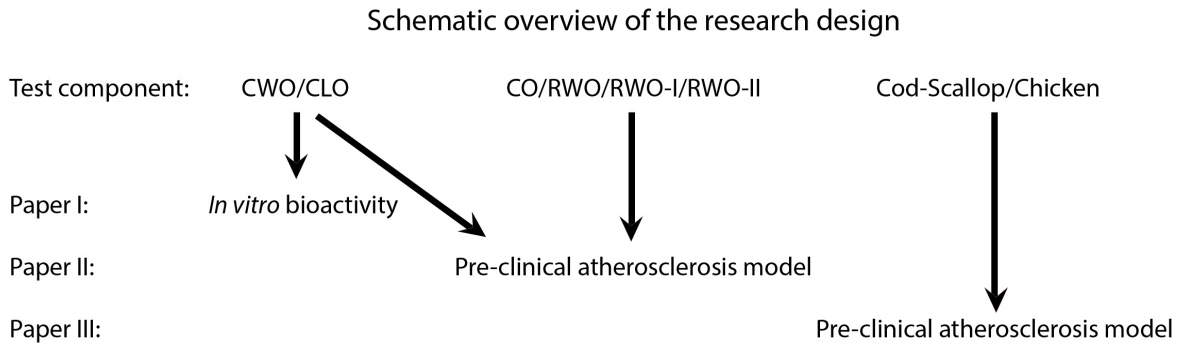


Figure 1 Schematic overview of the research design for paper I-III. CWO = cold-pressed whale oil, CLO = cod liver oil, CO = corn oil, RWO = refined whale oil, RWO-I = refined whale oil + extract I, RWO-II = refined whale oil + extract II.

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].

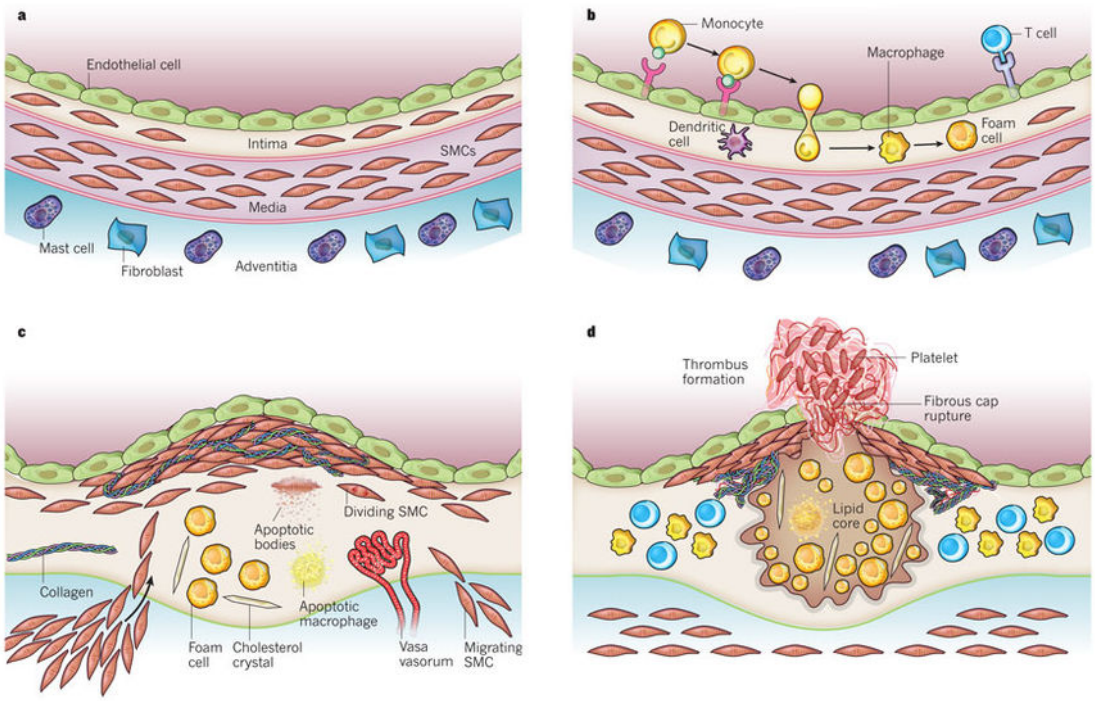


Figure 2 Atherosclerotic process by Libby et al. [30]. The process of atherosclerosis, from normal artery to thrombosis. **a)** The normal artery wall consists of three separate layers; the inner layer (tunica intima), middle layer (tunica media) and outer layer (tunica adventitia). Tunica intima is 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.

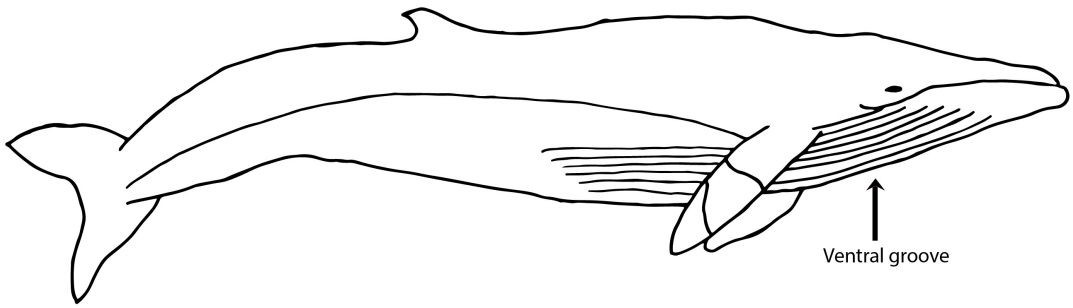


Figure 3 Illustration of the common minke whale and the ventral groove.

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.

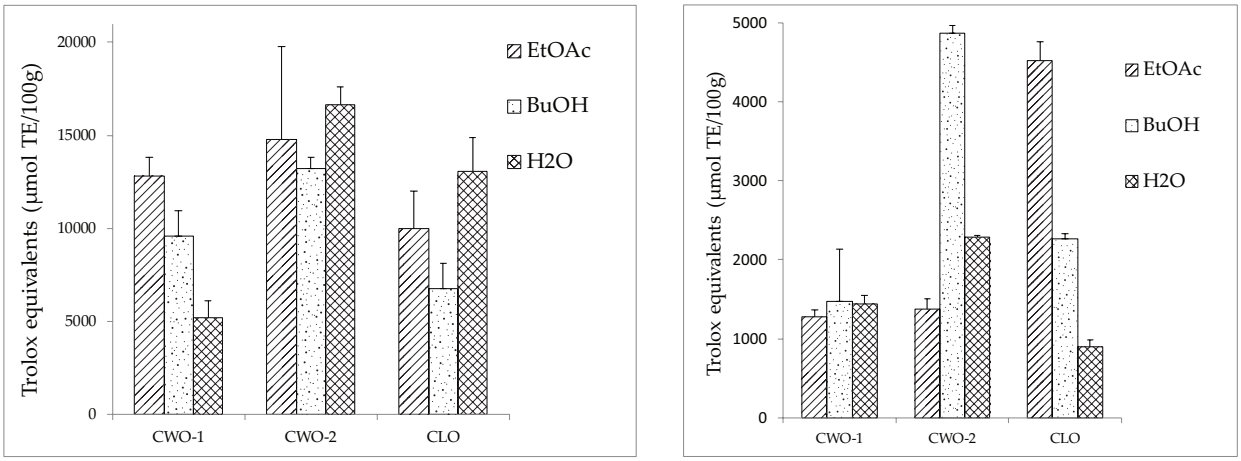
5 DISCUSSION OF MAIN RESULTS

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)

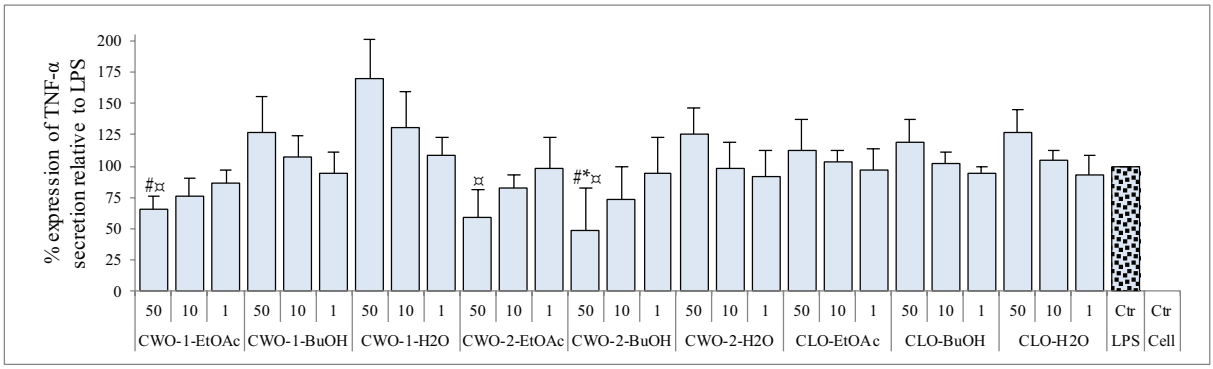
Figure 4 Antioxidative capacity retrieved from paper I. CWO-1, CWO-2 and CLO were extracted sequentially with EtOAc, BuOH and H_2O and the result are presented as Trolox equivalents (µmole TE/100g). (a) Oxygen Radical Absorbance Capacity (ORAC) assay (b) Ferric Reducing Antioxidant Power (FRAP) assay.

5.2 ANTI-INFLAMMATORY EFFECT ON CYTOKINE SECRETION

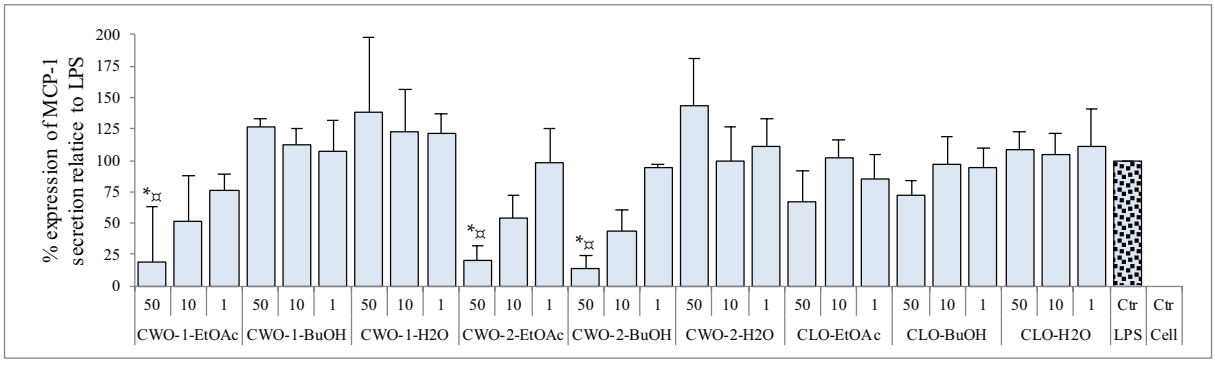
The macrophage like THP-1 cell line were established in 1980 [128] and have since been recognized as a suitable *in vitro* model to examine the regulatory and functional mechanisms of monocytes and macrophages in the cardiovascular system [129]. Anti-inflammatory effects were assessed based on the extracts´ 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.

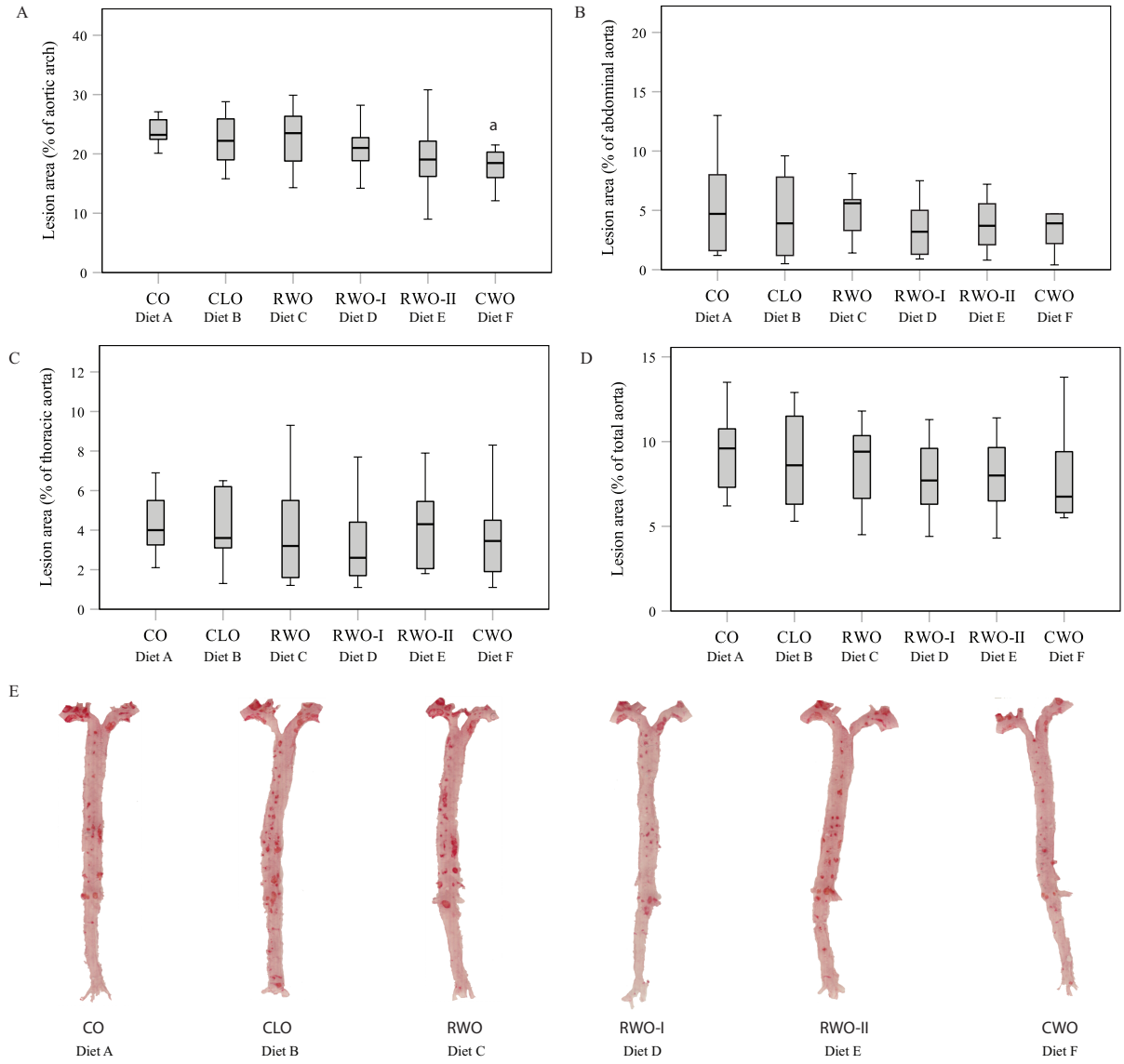


Figure 6 Atherosclerotic plaque burden retrieved from paper II. Female apolipoprotein E-deficient mice were, for 13 weeks, fed a high fat Western-type diets supplemented with different oils. (a-d) Diet A (n=11), diet B (n=9), diet C (n=11), diet D (n=11), diet E (n=12), diet F (n=10). **a** represents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

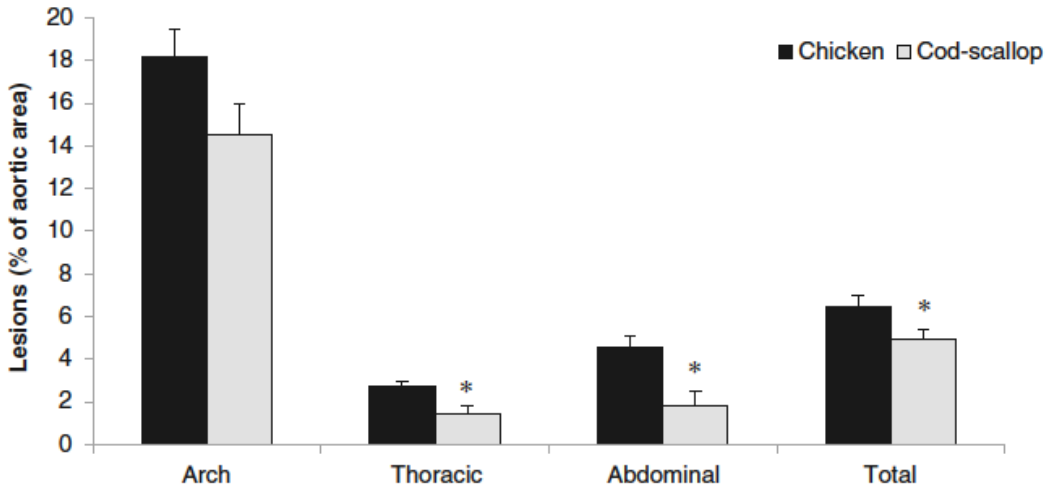


Figure 7 Atherosclerotic plaque burden retrieved from paper III. Female apolipoprotein E-deficient mice were fed high fat Western-type diets for 13 weeks with cod-scallop or chicken as the protein sources. *represent the significant difference between cod-scallop fed mice (n=10) and chicken-fed mice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

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Research Article

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

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Intake of long-chain omega-3 polyunsaturated fatty acids (LC-n3-PUFA) is commonly recognized to reduce cardiovascular disease (CVD). In previous studies, cold-pressed whale oil (CWO) and cod liver oil (CLO) were given as a dietary supplement to healthy volunteers. Even though CWO contains less than half the amount of LC-n3-PUFA of CLO, CWO supplement resulted in beneficial effects on anti-inflammatory and CVD risk markers compared to CLO. In the present study, we prepared virtually lipid-free extracts from CWO and CLO and evaluated the antioxidative capacity (AOC) and anti-inflammatory effects. Oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays were used to test the AOC, and the results indicated high levels of antioxidants present in all extracts. The anti-inflammatory effects of the extracts were tested with lipopolysaccharide- (LPS-) treated THP-1 cells, measuring its ability to reduce cytokine and chemokine secretion. Several CWO extracts displayed anti-inflammatory activity, and a butyl alcohol extract of CWO most effectively reduced TNF-α (50%, $p < 0.05$) and MCP-1 (85%, $p < 0.001$) secretion. This extract maintained a stable effect of reducing MCP-1 secretion (60%, $p < 0.05$) even after long-term storage. In conclusion, CWO has antioxidant and anti-inflammatory activities that may act in addition to its well-known LC-n3-PUFA effects.

1. Introduction

Atherosclerosis is a cardiovascular disease (CVD) characterized by lipid accumulation and chronic inflammation in the arteries. The atheromatous plaques accumulate over years within the intima of arteries and may ultimately rupture, resulting in atherothrombosis and myocardial infarction [1]. Even though the mortality rate from CVD has decreased in high-income countries during the last decades, CVD remains the leading cause of mortality worldwide [2]. Thus, novel therapies to reduce the atherosclerotic risk and to prevent severe adverse effects associated with prevailing treatments are still needed. In recent years, the inflammatory aspects of atherosclerosis have been thoroughly elucidated [3–5]. Several cytokines and chemokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), are important contributors in atherogenesis and atherosclerosis [6–10]. Consequently, novel anti-inflammatory components may contribute significantly in both prevention and therapeutic treatment of atherosclerosis. Intake of long-chain omega-3 polyunsaturated fatty acids (LC-n3-PUFA) is a recognized risk reducer of CVD due to the triacylglycerol-lowering and anti-inflammatory effects [11–13]. As LC-n3-PUFA is very susceptible to oxidation, it seems important to eat sufficient amounts of antioxidants to prevent lipid peroxidation [14]. Thus, combining LC-n3-PUFA with other anti-inflammatory agents may be an effective approach to reduce atherosclerosis. Bioprospecting for antioxidants and anti-inflammatory components has led to

an extended search in a wide range of marine species, mainly focusing on small organisms such as bacteria, fungi, and invertebrates [15]. In this context, larger marine mammals have received less attention. The minke whale (*Balaenoptera acutorostrata*) is an Atlantic finback whale regularly migrating to areas in the north where they feed on pelagic fish and crustaceans [16]. Minke whales have a thick layer of blubber [17] which is a vascularized hypodermal adipose tissue, vital for buoyancy, thermal insulation, and energy storage [18]. The blubber is a modified adipose tissue composed of adipocytes and connective tissue comprised of highly organized elastin and collagen fibers [19]. Intact blubber and oil extracted from blubber have been used in the diet in Arctic and Subarctic regions for centuries.

Previous dietary studies of cold-pressed oil from minke whale blubber (CWO) have indicated beneficial effects on CVD markers and improved an anti-inflammatory effect, also in comparison to cod liver oil (CLO) supplementation [20, 21]. Notably, the LC-n3-PUFA content in CWO was less than half when compared to CLO (10.3% versus 25.1%), indicating that the anti-inflammatory effects may rely on putative unknown components from blubber interacting with LC-n3-PUFA [21]. The objective in the present study was hence to elucidate possible *in vitro* antioxidative and anti-inflammatory effects of lipid-free extracts from CWO using biochemical assays and lipopolysaccharide-(LPS-) stimulated THP-1 cells.

2. Materials and Methods

2.1. Samples. Frozen fresh blubber from the ventral groove of the minke whale was provided from Ellingsen Seafood AS (Skrova, Norway).

2.2. Extraction. The blubber from the minke whale was grinded once before centrifugation at <2000 ×g (<40°C), and the oily top layer was collected (CWO-1). The remnant blubber was centrifuged again at the same speed, and again the oily top layer was collected (CWO-2). For comparison, commercially available CLO [22], rich in LC-n3-PUFA, was included. Each sample was further treated equally; 250 grams was extracted in 800 ml methanol/dichloromethane (1 : 1). The dichloromethane fraction containing most of the lipids was discarded, and residual dichloromethane in the methanol phase was removed by the use of a rotavapor. The remaining oil was removed by 3 × 200 ml heptane liquid-liquid extraction, and the methanol fraction was evaporated to almost dryness prior to addition of 100 ml water (dH_2O). Further evaporation removed residual methanol from the extract. After evaporation to almost dryness, the extract was once again redissolved in 100 ml dH_2O. The sample, now dissolved in water, was partitioned first with 3 × 200 ml ethyl acetate (EtOAc), then with 3 × 200 ml butyl alcohol (BuOH). The three extracts (EtOAc, BuOH, and H_2O) were subsequently evaporated using a rotavapor and, finally, to dryness under a steam of nitrogen. Extracts were stored at −20°C prior to further analyses, and stock solutions (10 mg/ml) were prepared in dH_2O and 5% dimethyl sulfoxide (DMSO).

2.3. Oxygen Radical Absorbance Capacity (ORAC). The antioxidative effect was measured with the ORAC assay [23]. Samples were mixed with the synthetic free radical generator 2,2′-azobis-2-methyl-propanimidamide dihydrochloride (AAPH, Sigma-Aldrich) and the oxidizable fluorescein sodium salt (number F6377, Sigma-Aldrich) at physiological pH. The water-soluble vitamin E equivalent Trolox was used as standard, and fluorescence decay, as a result of the radical attack, was measured at 485 and 520 nm (Spectramax Gemini EM fluorimeter, Molecular Devices, Sunnyvale, USA). The antioxidative capacity (AOC) was defined as the net difference between the areas under the fluorescence decay curves for the sample and blank, respectively. The results are presented as μmol Trolox equivalents (TE)/100 g oil. Extracts were measured at a concentration of 0.1 mg/ml sample.

2.4. Ferric Reducing Antioxidant Power (FRAP). The antioxidant activities of the extracts were also evaluated using FRAP assay [24]. Samples were incubated together with the FRAP reagents (ferric-tripyridyltriazine complex, pH 3.6) in a microtiter plate for 30 minutes. The intense blue color formed as a result of reduction to the ferrous-tripyridyltriazine complex was measured at 593 nm (Spectramax Gemini EM fluorimeter, Molecular Devices, Sunnyvale, USA). The AOC was determined as TE from the Trolox standard curve (0–1000 μM). The results are presented as μmol TE/100 g oil.

2.5. Differentiated THP-1 Cells. THP-1 (number TIB-202, ATCC) is a monocyte cell line derived from a patient with acute monocytic leukemia. The cell line grows in suspension; however, after treatment with phorbol 12-myristate 13-acetate (PMA), the cells differentiate into adherent macrophage-like cells [25]. The cells were maintained in RPMI-1640 (FG1385, Biochrom) media with 10% fetal bovine serum (FBS) (S0115, Biochrome) and 10 μg/ml gentamicin (A2712, Biochrome). Cells were incubated in 5% CO_2 atmosphere and 37°C for all THP-1-based experiments and subcultured every 3-4 days when cell concentration reached 8×10^5 viable cells. Every batch of THP-1 cells was evaluated with dose response against of LPS prior to use.

2.6. Anti-Inflammatory Screening Assay. Approximately 1×10^5 THP-1 cells were seeded out and differentiated with 50 ng/ml PMA (P1585, Sigma-Aldrich) in 96-well plates. After 48 hours of incubation, cells were washed with PBS and fresh RPMI (without PMA) was added. The plates were incubated for 24 hours before the addition of 90 μl fresh RPMI and 10 μl extracts in different concentrations (50 μg/ml, 10 μg/ml, and 1 μg/ml) to the respective wells. After a 1-hour incubation, LPS (L2630, Sigma-Aldrich) was added at a final concentration of 5 ng/ml to all wells except for the negative controls. The plates were then incubated for 6 hours and immediately frozen at −80°C. Negative, positive, and DMSO controls (0.05%) were included in every run.

2.7. ELISA

2.7.1. Tumor Necrosis Factor-Alpha (TNF-α). One day prior to the ELISA testing of TNF-α secretion, MaxiSorp 96F-well plates (Nunc) were coated with 2 μg/ml capture antibody

(eBioscience Inc., San Diego, CA, USA) and stored at 4°C, overnight. All incubations were at room temperature with shaking, and plates were washed with Tris-buffered saline (TBS) (pH 7.4, 0.05% Tween-20) between each step. Two hundred μl blocking buffer was added to each well before a 1-hour incubation. TNF-α samples were diluted at 1 : 4 and 1 : 10; TNF-α was added to each well before 2 hours of incubation. Biotin coupled anti-human antibody (eBioscience Inc., San Diego, CA, USA) was diluted in TBS + 1% bovine serum albumin (BSA) to 3 μg/ml and added to each well and subsequently incubated for 1 hour. Diluted ExtrAvidin®-Alkaline Phosphatase (Sigma-Aldrich) was added to each well prior to 30 min incubation. Finally, 100 μl pNPP substrate (Sigma-Aldrich, 1 mg/ml in 1 M buffer, pH 9.8) was added to each well and incubated for 45 min before the plates were read at 405 nm.

2.7.2. Monocyte Chemoattractant Protein-1 (MCP-1). MCP-1 secretion was analyzed with a quantitative sandwich enzyme-linked immunosorbent assay (Human CCL2, MCP-1, ELISA kit, 88-7399, eBioscience Inc., San Diego, CA, USA) according to the manufacturer's protocol.

2.8. Cell Viability. The cell viability was measured with the thiazolyl blue tetrazolium bromide (MTT) assay [26] in HT29 cells (HTB-38, ATCC). Approximately 1×10^5 cells were seeded in 96-well plates. After 48 hours of incubation, cells were washed with PBS before fresh media was added. To each well, 100 μl of extracts (final concentration of 50 μg), cell control, or deoxycholic acid (DCA, 500 μM as a positive control) was added. The plates were incubated for 24 hours, washed with PBS, and then 15 μl MTT (M2128, Sigma-Aldrich) was added to each well prior to 4 hours of incubating. One hundred μl of solubilizing buffer (2-propanol, hydrogen chloride, and Triton-x-100) was finally added to each well, and the plates were incubated for 4 hours before read at 570 nm.

2.9. Bio-Plex® Multiplex System (Bio Rad). This system enables detection and quantification of multiple analyses in a single sample volume. Three customized express plates (6-plex, 96-well flat bottom) preblended from human cytokine group 1 (TNF-α, MCP-1, IL-6, interleukin-10 (10), interferon gamma (IFN-γ), and RANTES) were used in this study. The assay was performed according to the manufacturer's protocol (Bio-Rad Laboratories Inc., Hercules, California, USA). These assays were performed after long-term storage (>4 years at −20°C) of the extracts to investigate whether the anti-inflammatory effects were stable through storage.

2.10. Thin-Layer Chromatography (TLC). Thin-layer chromatography was used to classify lipid classes. One μl (25 mg/ml in dichloromethane (DCM)) of the oil samples and 5 μl (1 mg/ml in DCM) extracts were analyzed on HP-TLC silica 60 plates (Merck Millipore, Billerica, Massachusetts, USA) in a solvent system of heptane:diethyl ether:acetic acid (70 : 30 : 1 *v*/*v*/*v*) to separate sample lipids. The plates were then treated with copper solution (10% cupric sulfate in 8% phosphoric acid), air dried for 10 minutes, and finally put in a cold oven and heated to 170°C. The standards used were 16-1A and 18-5A (Nu-Checkprep, Elysian, USA).

2.11. Statistical Methods. All of the statistical analyses were performed using IBM SPSS Statistics for Macintosh (released 22.0.0.0, SPSS Inc., Chicago, IL, USA). Values are presented as mean ± SD unless otherwise stated. The significance of differences was evaluated using one-way ANOVA, with Tukey's post hoc test. The difference at the level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Extraction Yield. Cold-pressed whale oil was separated into two consecutive samples (CWO-1 and CWO-2) after repeated grinding and centrifugation of the whale blubber. These samples were together with CLO further fractionated into three extracts (EtOAc, BuOH, and H_2O) each. There were large differences in extraction yields (expressed as dry matter yields produced from 250 g of the respective oils; mg/g). Dry weights of the extracts from CWO-1 were 0.33 mg/g (EtOAc), 2.07 mg/g (BuOH), and 1.58 mg/g (H_2O), and for the CWO-2 extracts, yields were 0.43 mg/g (EtOAc), 0.57 mg/g (BuOH), and 2.17 mg/g (H_2O). Contrary to the CWO extracts, the CLO extracts contained only small amounts of water-soluble polar components as reflected in the dry matter yields of 0.60 mg/g (EtOAc), 0.054 mg/g (BuOH), and 0.008 mg/g (H_2O). TLC (Figures 1(a) and 1(b)) confirmed that all of the extracts prepared from both CWO and CLO contained very few remaining lipids. It was also evident that the BuOH extracts from CWO-1 and CWO-2 contained very low amounts of residual free fatty acids and no triacylglycerol (TAG), diacylglycerol (DAG), or monoacylglycerol (MAG) (Figure 1(a), lanes 1–6). EtOAc extracts (from CWO-1 and CWO-2) contained some residual phospholipids, whereas the H_2O extracts contained no lipids. All the extracts from CLO (EtOAc, BuOH, and H_2O; Figure 1(b), lanes 4–6) contained trace amounts of lipids in the form of MAG and DAG, but no TAG. The whale blubber oil and cod liver oil both contained almost exclusively TAG (Figure 1(b), lanes 1 and 2). It is noteworthy that five times more extracts than oils were applied onto the TLC plates.

3.2. Antioxidative Capacity. Two methods, ORAC (Figure 2(a)) and FRAP (Figure 2(b)), were applied to determine the AOC of the extracts. In the ORAC assay, it was evident that CWO-2 had high AOC in all three extracts (CWO-2-EtOAc, CWO-2-BuOH, and CWO-2-H_2O extracts), and correspondingly, CWO-2 had the highest total AOC. In the FRAP assay, on the other hand, CWO-2-BuOH together with CLO-EtOAc displayed the highest AOC.

3.3. Anti-Inflammatory Effect on Cytokine Secretion in LPS-Treated THP-1 Cells. The anti-inflammatory effects of the extracts were studied using LPS-stimulated THP-1 macrophage-like cells. Inhibition of LPS-induced TNF-α and MCP-1 production (Figures 3(a) and 3(b), resp.) was most pronounced for CWO-2-BuOH. The extracts were applied at 3 different concentrations, and a dose-dependent

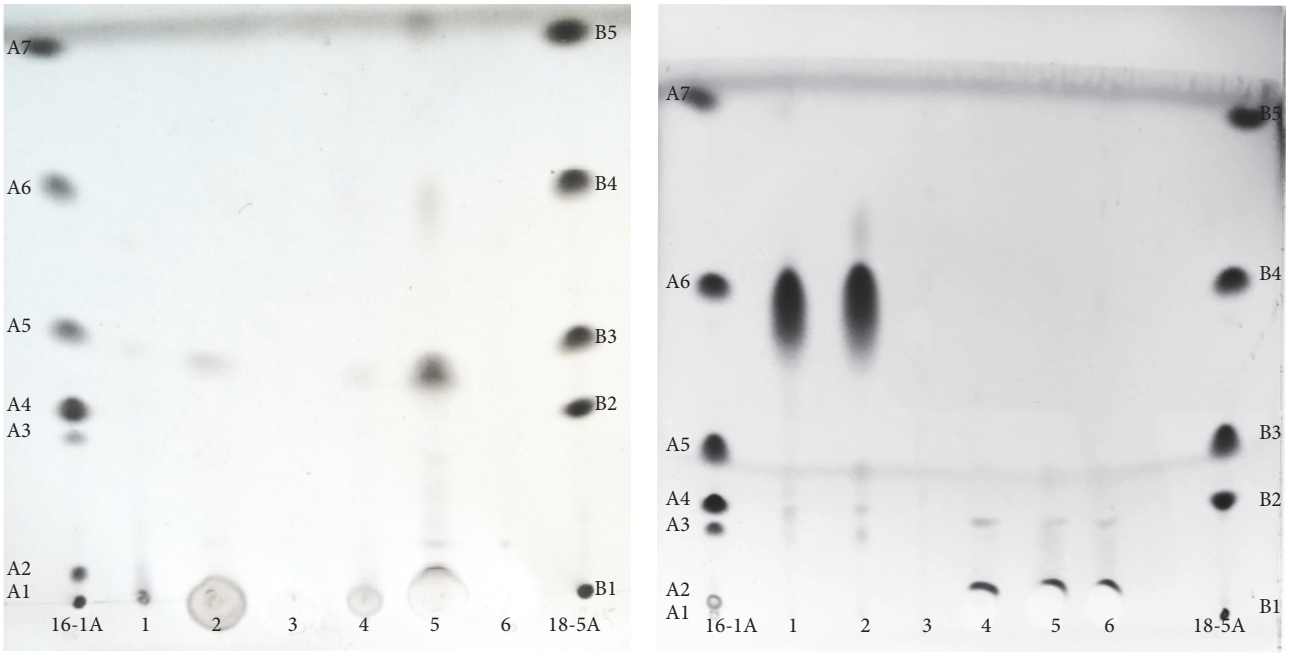

Figure 1: HP-TLC of oils (prior to extraction) and extracts. Fatty acid standards: 16-1A containing phospholipids (A1), monoacylglycerol (A2), diacylglycerol (A3), cholesterol (A4), free fatty acids (A5), triacylglycerol (A6), and cholesteryl ester (A7). 18-5A containing phospholipids (B1), cholesterol (B2), free fatty acids (B3), triacylglycerol (B4), and cholesteryl ester (B5). (a) Extracts: CWO-1 EtOAc (1), CWO-1 BuOH (2), CWO-1 H_2O (3), CWO-2 EtOAc (4), CWO-2 BuOH (5), and CWO-2 H_2O (6). (b) Oils before extraction: CLO (1) and CWO (2) empty (3) extracts: CLO-1 H_2O (4), CLO-1 BuOH (5), and CLO EtOAc (6). HP-TLC = high performance thin-layer chromatography; CWO = cold-pressed whale oil; CLO = cod liver oil; EtOAc = ethyl acetate; BuOH = butyl alcohol.

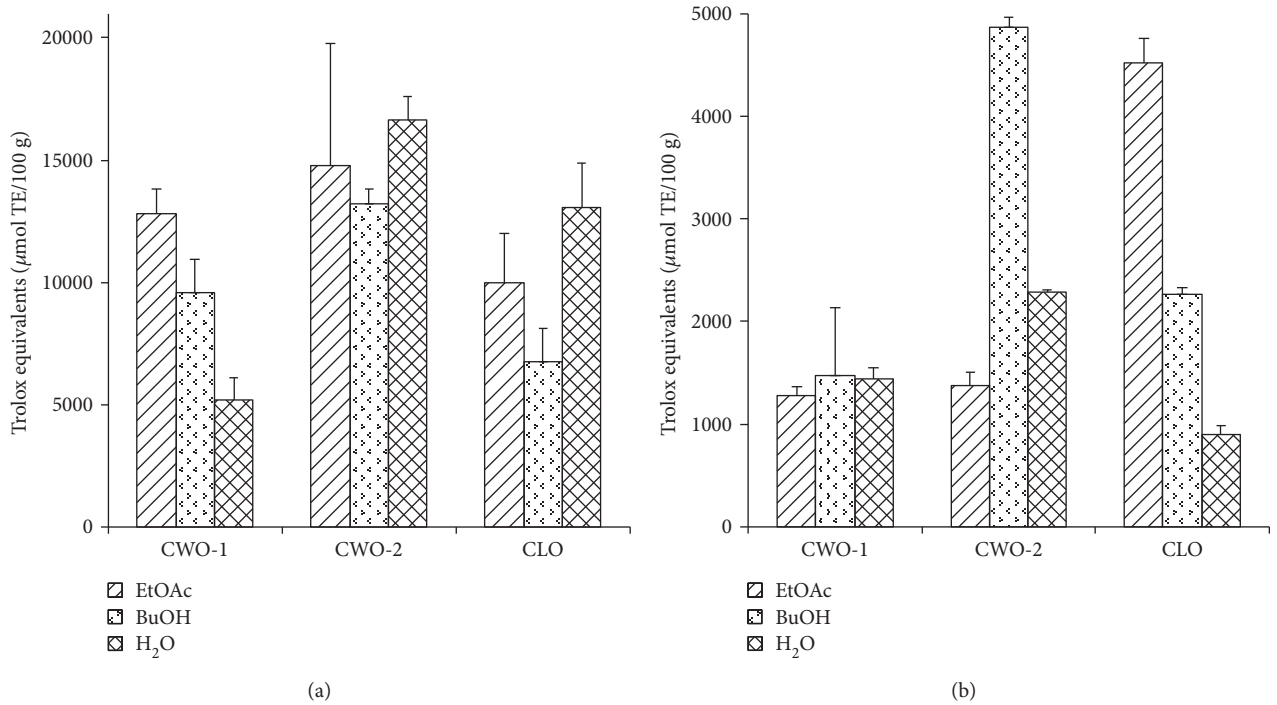

Figure 2: Antioxidative capacity in the extracts. CWO-1, CWO-2, and CLO which were sequentially extracted using EtOAc, BuOH, and water. The results are shown as Trolox equivalents (μmol TE/100 g). (a) ORAC assay with extract concentrations of 0.1 mg/ml. (b) FRAP assay with extract concentrations of 10 mg/ml. CWO = cold-pressed whale oil; CLO = cod liver oil; EtOAc = ethyl acetate; BuOH = butyl alcohol; ORAC = oxygen radical absorbance capacity; FRAP = ferric reducing antioxidant power.

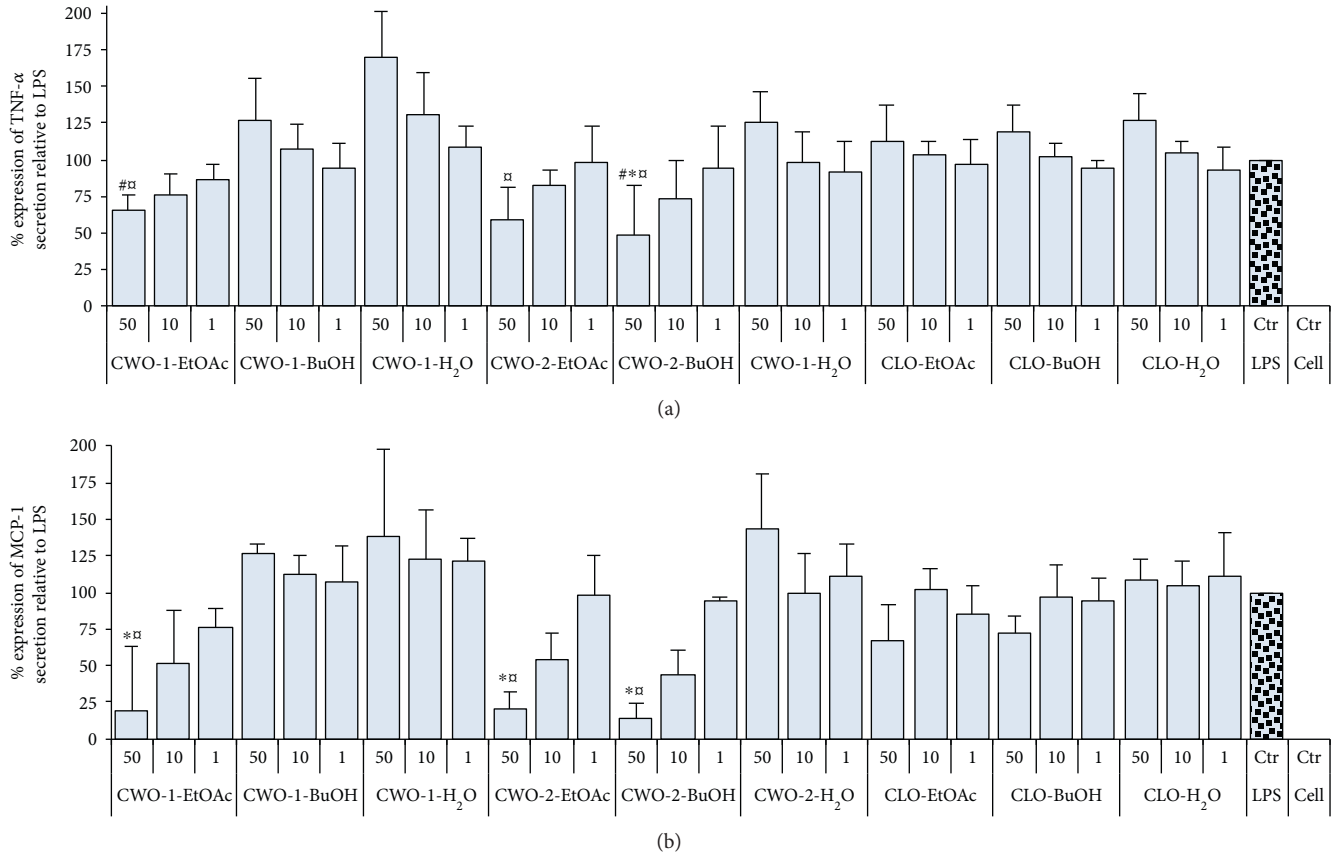

Figure 3: ELISA results for relative response of TNF-α and MCP-1 in LPS-treated THP-1 cells. Each extract was tested at three different concentrations (50 μg/ml, 10 μg/ml, and 1 μg/ml), and the results are presented as mean with SD displayed as positive bars ($n = 3$). (a) TNF-α secretion relative to control. $^{*}p < 0.05$ compared to LPS control. $^{\#}p < 0.05$ compared to CLO-BuOH 50 μg/ml. $^{¤}p < 0.05$ compared to CLO-H_2O 50 μg/ml. (b) MCP-1 secretion relative to control. $^{*}p < 0.001$ compared to LPS control. $^{¤}p < 0.001$ compared to CLO-H_2O 50 μg/ml. TNF-α = tumor necrosis factor-alpha; MCP-1 = monocyte chemoattractant protein-1; LPS = lipopolysaccharide; CWO = cold-pressed whale oil; CLO = cod liver oil; EtOAc = ethyl acetate; BuOH = butyl alcohol.

inhibition of TNF-α and MCP-1 secretion was observed for this extract. When applying a dose of 50 μg/ml (CWO-2-BuOH), TNF-α and MCP-1 production were reduced by 50% ($p < 0.05$) and 85% ($p < 0.001$), respectively. The extracts CWO-1-EtOAc and CWO-2-EtOAc also inhibited MCP-1 secretion dose dependently, and with a final extract concentration of 50 μg/ml, the MCP-1 secretion was reduced by approximately 80% ($p < 0.001$, Figure 3(b)). A nonsignificant tendency of the reduction of TNF-α secretion was also observed for CWO-1-EtOAc and CWO-2-EtOAc (Figure 3(a)). None of the extracts affected LPS-induced IL-1β production (results not shown). Negative controls with cell media or vehicle (0.05% DMSO) did not stimulate the release of any of the tested cytokines. Cells incubated with extracts made from CLO were also analyzed; however, no effects were observed on TNF-α (Figure 3(a)), MCP-1 (Figure 3(b)), or IL-1β secretion (results not shown). When comparing the effects of treatment of THP-1 cells with all the extracts from CWO-1 and CWO-2 with the corresponding extracts from CLO, lower LPS-induced TNF-α secretion was observed for CWO-1-EtOAc ($p < 0.05$) and CWO-2-BuOH ($p < 0.005$) relative to treatment with CLO-BuOH (50 μg/ml for all extracts, Figure 3(a)). The levels of LPS-induced TNF-α release ($p < 0.05$) after treatment of the THP-1 cells with 50 μg/ml of CWO-1-EtOAc, CWO-2-EtOAc, or CWO-2-BuOH were lower compared to LPS-induced TNF-α production after treatment with 50 μg/ml CLO-H_2O (Figure 3(a)). There was a similar pattern for MCP-1, as LPS-induced MCP-1 release from THP-1 cells treated with extracts (at 50 μg/ml) from CWO-1-EtOAc, CWO-2-EtOAc, or CWO-2-BuOH was different ($p < 0.001$) compared to treatment with 50 μg/ml of CLO-H_2O extract. The cell viability was tested using the MTT assay, and none of the CWO-1 or CWO-2 extracts affected the cell viability (Figure 4).

3.4. Effect of Storage on Anti-Inflammatory Activity of the Extracts. The effects of the extracts after long-term storage (>4 years at −20°C) on LPS-induced production of TNF-α, MCP-1, IL-6, IL-10, and RANTES were also assessed (Table 1). CWO-2-BuOH (50 μg/ml) inhibited MCP-1 production in LPS-treated THP-1 cells with more than 60% ($p < 0.05$). In cells treated with CWO-1-EtOAc, both LPS-induced TNF-α and MCP-1 were reduced, whereas secretion of anti-inflammatory IL-10 was increased in cells treated with CWO-2-EtOAc ($p = 0.054$, Table 1). Secretion of LPS-

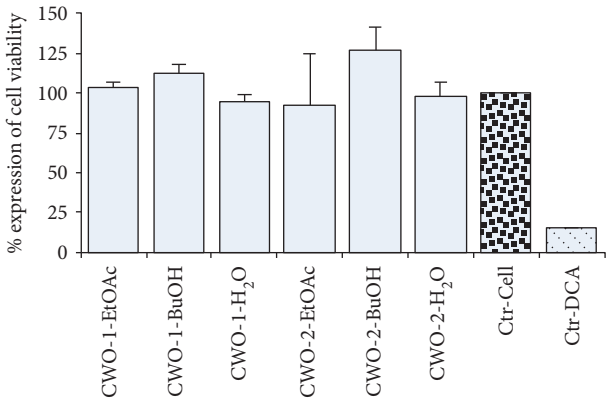

Figure 4: MTT results for cell viability of CWO-1 and CWO-2 extracts. Each extract was tested at 50 μg/ml, and the results are presented as mean with SD displayed as positive bars ($n = 3$). The cell viability was calculated relative to the cell control (cell media), and 500 μM DCA was included as a positive control. MTT = thiazolyl blue tetrazolium bromide; CWO = cold-pressed whale oil; EtOAc = ethyl acetate; BuOH = butyl alcohol; DCA = deoxycholic acid.

induced RANTES was not affected by treatment with any of the extracts (Table 1). CWO-2-BuOH (50 μg/ml) inhibited IL-6 production ($p = 0.058$) compared to CLO-BuOH (50 μg/ml), and CWO-2-EtOAc (50 μg/ml) increased IL-10 production ($p = 0.077$) compared to CLO-EtOAc (50 μg/ml, Table 1).

4. Discussion

Previous intervention studies comparing intake of CWO with intake of CLO indicated that CWO has anti-inflammatory effects not observed after intake of CLO [20, 21]. Epidemiological studies during the 1970s indicated anti-inflammatory and antioxidative effects of ingestion of blubber based on the low incidence of CVD among the indigenous people in Greenland [27, 28]. It is however important to notice that the Inuit consumed mainly fish in addition to meat and blubber from seals and whales. It has also been claimed that the prevalence of CVD was underestimated in this population [29, 30].

In our study, most of the lipids, and thus the fatty acids, were removed prior to *in vitro* testing to investigate whether blubber contained compounds that could act in synergy or in addition to the anti-inflammatory effects previously ascribed to the LC-n3-PUFA. To prevent the destruction of putative temperature-labile lipophilic antioxidants present in the whale blubber, extracts were prepared from CWO at low temperatures (<40°C). After removal of the most lipophilic parts of the oil samples, extracts with different polarity were prepared using EtOAc, BuOH, and water as extraction solvents. Thin-layer chromatography indicated that the BuOH extracts from CWO samples contained very low amounts of residual free fatty acids and trace amounts of TAG, DAG, or MAG. The CWO-1-EtOAc and CWO-2-EtOAc extracts contained some residual lipids, whereas the H_2O extracts contained no lipids. For the extracts prepared from CLO, the TLC analysis showed that all these extracts contained very low amounts of free fatty acids, TAG, DAG, and MAG.

The total AOC observed in the extracts in this work may be considered high compared to other organic materials tested with FRAP and ORAC assays [31, 32]. The high AOC indicates that the tested extracts contain antioxidants that may protect against reactive oxygen species *in vivo*. ORAC assay is regarded more physiologically relevant than FRAP due to pH and temperature; however, it is important to emphasize that both these assays are simplified methods to measure AOC. These assays measure quite different mechanisms and are not fully comparable, and it was not surprising that the CWO-2-BuOH displayed high reducing power in the FRAP assay and lower ORAC activity. However, the total AOC from each sample shows the same ranking order (CWO-2 > CLO > CWO-1). Another important aspect is the dry matter yield. Being 5-6 times lower in CLO compared to CWO-2 and CWO-1, this results in lower total amount of antioxidants in CLO. Despite the simplicity of the assays used, these results provided the fundament for the investigation of anti-inflammatory effects.

Several of the extracts displayed potent and dose-dependent anti-inflammatory activity demonstrated through the reduction of LPS-induced production of chemokine (MCP-1) and cytokine (TNF-α). The most pronounced inhibition of TNF-α and MCP-1 was observed in cells treated with the CWO-2-BuOH extract, but treatment with CWO-1-EtOH and CWO-2-EtOAc also inhibited TNF-α production. It is possible that the concentrations (50, 10, and 1 μg/ml) used in the present study might have been too low to reveal the full anti-inflammatory potential of the extracts.

All the extracts that inhibited LPS-induced TNF-α also inhibited release of MCP-1. However, the inhibiting effects on LPS-induced MCP-1 were much more pronounced, as MCP-1 levels were reduced 80% to 85% by these extracts. MCP-1 is an important contributor for atherosclerosis, and therefore potent and specific inhibitors of MCP-1 may be attractive drug candidates for the prevention of atherosclerosis. Contrary to the extracts produced from CWO, none of the CLO extracts affected any of the investigated cytokines or chemokines. The levels of secreted IL-1β were unchanged after treatment with all the extracts, apparently due to the different regulation mechanisms for IL-1β compared to the other cytokines and chemokines tested in this study [33].

The nature of many bioprospecting projects involves sample collection at very remote locations, before processing, extracting, and further investigation at different laboratories. This actualized a desire to determine if the putative bioactive compounds were sufficiently robust to be discovered with this kind of approach. The effects of long-term storage (>4 years at −20°C) on the anti-inflammatory activity in our extracts were investigated with a multiplex assay including MCP-1, TNF-α, IL-10, IL-6, RANTES, and IFN-γ. The anti-inflammatory activity was preserved for CWO-2-BuOH reducing MCP-1 secretion compared to LPS control. Interestingly, CWO-2-EtOAc increased the IL-10 production compared to both LPS control and CLO-EtOAc. IL-10 is considered to have anti-inflammatory capacity *in vivo* and is known to hold a critical role as a feedback regulator

Table 1: Cytokine responses in LPS-induced THP-1 cells treated with different extracts result in relative % compared to LPS control. $^{*}p < 0.05$ compared to LPS control, $^{**}p = 0.054$ compared to LPS control and $p = 0.077$ compared to CLO-EtOAc 50 μg/ml, and $^{\#}p = 0.058$ compared to CLO-BuOH 50 μg/ml. TNF-α: tumor necrosis factor-alpha; MCP-1: monocyte chemoattractant protein-1; IL-6: interleukin-6; IL-10: interleukin-10; RANTES: regulated on activation, normal T cell expressed, and secreted; LPS: lipopolysaccharide; CWO: cold-pressed whale oil; CLO: cod liver oil; EtOAc: ethyl acetate; BuOH: butyl alcohol.

Extracts	Extract conc. (μg/ml)	IL-6 (% of LPS)	IL-10 (% of LPS)	MCP-1 (% of LPS)	RANTES (% of LPS)	TNF-α (% of LPS)
CWO-1-H_2O	50	282 ± 92	212 ± 208	97 ± 17	126 ± 26	136 ± 81
	10	163 ± 40	92 ± 29	125 ± 23	141 ± 8	142 ± 67
	1	120 ± 20	99 ± 115	115 ± 18	128 ± 6	111 ± 38
CWO-1-BuOH	50	117 ± 3	102 ± 29	91 ± 2	110 ± 26	92 ± 37
	10	60 ± 53	ND	68 ± 56	91 ± 73	78 ± 65
	1	95 ± 17	92 ± 21	103 ± 15	116 ± 2	102 ± 39
CWO-1-EtOAc	50	85 ± 9	219 ± 106	58 ± 7	101 ± 27	60 ± 14
	10	90 ± 3	99 ± 35	100 ± 8	115 ± 10	90 ± 30
	1	106 ± 19	115 ± 25	117 ± 23	117 ± 12	104 ± 41
CWO-2-H_2O	50	170 ± 49	166 ± 121	100 ± 11	109 ± 22	114 ± 50
	10	96 ± 7	91 ± 22	104 ± 9	127 ± 9	104 ± 22
	1	99 ± 16	95 ± 19	102 ± 13	137 ± 21	102 ± 36
CWO-2-BuOH	50	21 ± 12$^{\#}$	160 ± 43	37 ± 13*	80 ± 25	42 ± 18
	10	71 ± 13	123 ± 35	105 ± 11	126 ± 24	91 ± 33
	1	91 ± 31	ND	108 ± 35	112 ± 32	103 ± 57
CWO-2-EtOAc	50	105 ± 34	272 ± 133**	53 ± 11	98 ± 28	101 ± 65
	10	95 ± 17	137 ± 68	96 ± 12	141 ± 45	96 ± 38
	1	106 ± 16	112 ± 19	110 ± 12	135 ± 22	107 ± 42
CLO-H_2O	50	141 ± 47	145 ± 99	91 ± 10	99 ± 11	89 ± 36
	10	112 ± 38	ND	99 ± 18	115 ± 25	97 ± 45
	1	121 ± 44	ND	115 ± 24	117 ± 24	101 ± 49
CLO-BuOH	50	121 ± 23	100 ± 21	80 ± 6	104 ± 29	83 ± 37
	10	112 ± 32	ND	104 ± 14	109 ± 8	101 ± 44
	1	108 ± 24	ND	103 ± 24	133 ± 31	110 ± 50
CLO-EtOAc	50	113 ± 18	ND	90 ± 16	105 ± 14	101 ± 49
	10	122 ± 43	ND	111 ± 21	120 ± 12	104 ± 45
	1	101 ± 21	ND	110 ± 29	115 ± 25	100 ± 48
LPS	Ctr	100	100	100	100	100
Cell	Ctr	0	5 ± 4	2 ± 2	3 ± 3	0
DMSO	Ctr	0	5 ± 5	1 ± 1	4 ± 3	0

of a wide range of immune responses [34]. Secreted RANTES levels were not affected by any of the extracts, while CWO-2-BuOH downregulated the MCP-1 secretion. Different signaling pathways activate the production of RANTES and MCP-1 after LPS/Toll-like receptor 4 stimulation. RANTES belongs to the MyD88-dependent pathway [35, 36] whereas MCP-1 belongs to the MyD88-independent pathway [37]. In this study, virtually all of the hydrophobic heptan-dissolvable lipids were removed during the extraction process to establish anti-inflammatory activities independent of LC-n3-PUFA. Since triacylglycerol has also been found to downregulate MCP-1 expression in THP-1 cells [38], additional effects could be expected if the fatty acids were included in the extracts. During the refining process of commercial CLO, the oils are subjected to high temperature treatment, which may cause degradation and loss of putative anti-inflammatory compounds. This might explain the absence of anti-inflammatory activity in the CLO extracts despite the presence of antioxidative capacities.

5. Conclusion

This study has demonstrated that CWO contains antioxidants and anti-inflammatory activities that are not related to its content of LC-n3-PUFA. This indicates that there are unidentified extractable anti-inflammatory compounds present in whale oil yet to be discovered. To investigate the anti-inflammatory effects further, the putative bioactive components need to be isolated and CWO (still containing the fatty acids) should be evaluated *in vivo* in chronic inflammatory animal models.

Disclosure

The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Karl-Erik Eilertsen, Svein Kristian Stormo, Mari Johannessen Walquist, and Bjarne Østerud conceived and designed the experiments. Mari Johannessen Walquist, Svein Kristian Stormo, and Ida-Johanne Jensen performed the experiments. Mari Johannessen Walquist and Karl-Erik Eilertsen analyzed the data. Mari Johannessen Walquist and Karl-Erik Eilertsen wrote the paper. All authors have read and approved the final manuscript.

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Paper II

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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

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Abstract

Background: Long-chain n3-polyunsaturated fatty acids (LC n3-PUFA) are well known for their anti-inflammatory activity and their impact on cardiovascular disease. Cold-pressed whale oil (CWO) has half the amount of LC n3-PUFA compared to cod liver oil (CLO). Still, there has been observed more pronounced beneficial effects on cardiovascular disease markers from intake of CWO compared to intake of CLO in human intervention studies. Extracts from CWO deprived of fatty acids have also been shown to display antioxidative and anti-inflammatory effects in vitro. The aim of this study was to investigate whether intake of a high-fat Western-type diet (WD) supplemented with CWO would prevent the development of atherosclerotic lesions in apolipoprotein E-deficient ($ApoE^{-/-}$) mice.

Methods: Seventy female $ApoE^{-/-}$ mice were fed a WD containing 1% CWO, CLO or corn oil (CO). Atherosclerotic lesion formation, body and tissue weights, hepatic gene expression together with serum levels of LDL/VLDL-cholesterol, ox-LDL, total antioxidant status and various serum cardiovascular disease/proinflammatory markers were evaluated. Statistical analyses were performed using SPSS, and Shapiro-Wilk's test was performed to determine the distribution of the variables. Statistical difference was assessed using One-Way ANOVA with Tukeys' post hoc test or Kruskal-Wallis test. The hepatic relative gene expression was analysed with REST 2009 (V2.0.13).

Results: Mice fed CWO had less atherosclerotic lesions in the aortic arch compared to mice fed CO. Levels of LDL/VLDL-cholesterol and ox-LDL-cholesterol were also markedly reduced whereas total antioxidant levels were enhanced in mice fed CWO compared to CO-fed mice. In addition, CWO-fed mice gained less weight and several hepatic genes involved in the cholesterol metabolism were up-regulated compared to CO-fed mice.

Conclusion: In the present study mice fed a WD supplemented with 1% CWO had reduced formation of atherosclerotic lesions in the aortic arch, reduced serum LDL/VLDL-cholesterol and ox-LDL-cholesterol, increased serum total antioxidant status and reduced body weight compared to mice fed a WD supplemented with 1% CO.

Keywords: Atherosclerosis, Plaque, Lesions, *Balaenoptera acutorostrata*, Whale oil, Blubber, Gene expression, LDL-cholesterol, VLDL-cholesterol

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Background

Atherosclerosis is characterized by arterial lipid lesions in the intima of large arteries and the illness may be silent or symptomless for decades. Yet, atherosclerosis is the major underlying cause of several other cardiovascular diseases (CVD) such as unstable angina, myocardial infarction and stroke. Years of lipid accumulation in the vascular wall cause plaque formation and narrowing of the lumen. Plaque rupture may eventually lead to acute atherothrombosis preventing adequate blood flow to lung, heart and brain tissue [1, 2]. The link between atherosclerosis and inflammation has been comprehensively elucidated [3–7], and several cytokines and chemokines are key contributors in atherosclerotic progression [8–11]. The anti-inflammatory effects of long-chain n3-polyunsaturated fatty acids (LC n3-PUFA) are well known. Especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and how they contribute to mitigate atherosclerotic progression in coronary patients [12–17]. Oily fish is a good dietary source of EPA and DHA. Fish also contain other constituents such as proteins, amino acids, peptides and bioactive compounds that may contribute to the beneficial effects observed after fish intake [18–20].

The common minke whale (*Balaenoptera acutorostrata*) feeds on crustaceans and pelagic fish in the North Atlantic [21], and their thick layer of blubber is vital for thermal insulation, buoyancy and energy storage [22]. Blubber has been an important part of the diet to indigenous people in Arctic and Subarctic regions for centuries. Two decades ago, cold-pressed whale oil (CWO) and cod liver oil (CLO) were given as dietary supplements to healthy individuals as part of a larger study [23, 24]. The results indicated that the CWO group had beneficial effect on CVD markers and improved inflammatory effect, also when compared to the CLO group. The fatty acid (FA) composition of CWO and CLO differ in the amount of LC n3-PUFA. Whale blubber has 10.3% LC n3-PUFA, including 3.3% EPA, 1.7% docosapentaenoic acids (DPA) and 4.7% DHA while CLO has 25.1% LC n3-PUFA, including 9.5% EPA, 1% DPA and 13.5% DHA in CLO [24]. Recently, we demonstrated in an in vitro study with CWO deprived of fatty acids, that CWO contained extractable antioxidants and had anti-inflammatory activities associated with hitherto unidentified compound(s) [25].

In the present study, we investigated whether intake of a high-fat Western-type diet (WD) supplemented with CWO or refined whale oil (RWO) in combination with extracts from whale blubber would prevent the development of atherosclerotic lesions in Apolipoprotein E-deficient ($ApoE^{-/-}$) mice.

Methods

Experimental animals and housing

Seventy-two pathogen-free female $ApoE^{-/-}$-mice (B6.129P2-*Apoe*$^{tm1\ Unc}$ N11) were purchased from Taconic (Taconic M&B, Ry, Denmark). After arrival at the local animal facility all mice were earmarked and randomly allocated into 6 groups ($n = 12$) with equal numbers of cages per intervention ($n = 4$ cages/diet). Due to weight loss during the one-week acclimatization, two mice were excluded ($n = 11$ in the RWO-I and CWO groups). At the start of the experiment, the mice were 6 weeks of age with a body weight range of 16 to 21 g. Mice provided with water and pelleted feed ad libitum for 13 weeks were kept in ventilated cages placed in the same room in a conventional laboratory animal unit. The temperature and relative humidity were 21 °C and 55% on a 12-h day/night cycle (light: 0600 to 1800 h). The cages and bedding were changed once a week. At the end of the study all mice were feed-deprived for 3 h prior to euthanizing by carbon dioxide inhalation. Blood was drawn by cardiac puncture and serum was prepared and frozen at − 80 °C. Cardiac, hepatic, renal, splenic and adipose tissues were dissected out, weighed, snap frozen and stored at − 80 °C.

Preparation of dietary oils

Freshly frozen blubber from common minke whale was provided by Ellingsen Seafood AS (Skrova, Norway). The blubber was ground once before centrifugation at < 2000×g (< 40 °C). After centrifugation the oily top-layer, hereafter referred to as CWO in this study, was collected and 250 g CWO was extracted in 800 ml methanol/dichloromethane (1:1). After phase separation, most of the lipids (oil) was in the fraction containing dichloromethane. The better part of the dichloromethane was removed by a rotary evaporator and the remaining solvent was removed during nitrogen flushing for 48 h. After extraction of the CWO, the oil that was left is referred to as RWO. For the methanol phase, containing more polar compounds extracted from CWO, residual dichloromethane and oil were removed with a rotary evaporator followed by 3 × 200 ml heptane liquid-liquid extraction. Subsequently, the methanol in this fraction was evaporated to almost dryness and was finally removed by flushing the sample with nitrogen for 48 h (extract I). The remnant of whale blubber after the initial removal of oil top layer was next extracted according to the same protocol as for CWO. Due to the low level of oil in the cold pressed blubber, refined whale oil was not collected from this dichloromethane fraction. Extract II (whale blubber extract) was also flushed with nitrogen for 48 h to remove all traces of solvents. Oils and extracts were flushed with nitrogen and stored at − 20 °C prior to further analyses. Commercial CLO was bought from Orkla Health [26] whereas CO was bulk oil provided by the diet manufacturer.

Experimental diets

The mice were fed six different high-fat WD (modified from EF D-12079, *ssniff Spezialdiäten GmbH*). The six diets were supplemented with 1% of six different PUFA-rich

oils as indicated in Table 1. Diet A was a control diet containing 1% CO, diet B contained 1% commercial CLO [26], diet C contained 1% RWO, diet D contained 1% RWO-I, diet E contained 1% RWO-II, and diet F contained 1% CWO. Apart from the PUFA-source the diets were identical, however to compensate for the higher cholesterol content of CLO compared to the other dietary oils used in this study, a smaller amount of cholesterol was added to the CLO diet (1.47 g/kg vs 1.5 g/kg for the rest of the diets, Table 1). The experimental diets were stored at − 20 °C and the feed was changed every week.

Analysis of atherosclerotic plaque

Immediately after blood drainage, all mice were perfused through the left ventricle with sterile saline (0.9%), until no residual blood was apparent in the perfusate (approximate 5 min perfusion). The entire aorta (proximal ascending the aorta to bifurcation of the iliac arteries) was cleaned in situ of periadventitial fat, dissected and fixed in 1% paraformaldehyde solution. Finally, the aorta was stained by Oil Red O staining, opened longitudinally and *en face*-mounted on slides as previously described [27]. After 48 h rest, the slides were scanned with a high-resolution scanner. The lesion areas were evaluated using ImageJ software [28] and the extent of atherosclerosis was reported as the percentage of the total area of a given aortaor an aortic region occupied by atherosclerotic lesions.

Total RNA extraction

After perfusion of the mice, livers were removed, weighed and frozen in liquid nitrogen before storage at − 80 °C until extraction. One hundred mg liver tissue was homogenized in 1 ml Trizol (Life Technologies) by bead milling (Precellys 24, Bertin Technologies). The samples were incubated on ice for 30 min followed by 20 min centrifugation at 12000×g at 4 °C. The samples were precipitated overnight with isopropanol and centrifuged for 21,000×g for 30 min at 4 °C. Pellets were dissolved in RNA storage solutions and RNA was stored at − 80 °C until further processing. Total RNA concentration was measured using Qubit fluorometer 1.0 (Life Technologies) and the quality tested with Agilent 2100 Bioanalyzer (Agilent Technologies, Inc). The RNA Integrity Factor was of 7.6–9.3 for the samples used.

Reverse transcription and quantitative real-time PCR

High capacity cDNA Reverse Transcription kit (4,368,813, Applied Biosystems) was used to make 3 triplicates of reverse transcriptase (50 ng total RNA in 20 μl). Quantitative RT-PCR was used to analyse 4 μl cDNA per 20 μl reaction using TaqMan® Fast Universal PCR Master mix (4,352,042, Applied Biosystems) and with predesigned TaqMan® Gene Expression assays (Additional file 1: Table S1). The 96-wells plates were run at ABI Prism 7500 Fast cycler (Applied Biosystems) using the pre-set amplification profile (95 °C 20s, 40 × 95 °C 3 s and 60 °C 30s). The most stably expressed endogenous reference genes, Hypoxanthine-

Table 1 Content of diets g/kg

	CO Diet A	CLO Diet B	RWO Diet C	RWO-I Diet D	RWO-II Diet E	CWO Diet F
Corn oil	10					
Cod liver oil		10				
Refined whale oil			10			
Refined whale oil + extract I				10		
Refined whale oil + extract II					10	
Cold-pressed whale oil						10
Casein	195	195	195	195	195	195
Corn starch	50	50	50	50	50	50
Maltodextrin. 10 DE	99.4	99.4	99.4	99.4	99.4	99.4
Sucrose	340	340	340	340	340	340
Celullose powder	50	50	50	50	50	50
DL-Methionine	3	3	3	3	3	3
AIN mineral premix	35	35	35	35	35	35
Vitamin premix	10	10	10	10	10	10
Calcium carbonate	4	4	4	4	4	4
Choline Cl	2	2	2	2	2	2
Butylated hydroxytoluene	0.1	0.1	0.1	0.1	0.1	0.1
Cholesterol	1.5	1.47	1.5	1.5	1.5	1.5
Butter fat	200	200	200	200	200	200

guanine phosphoribosyltransferase 1 *(Hprt1)* and TATA-Box Binding Protein (*Tbp)*, were selected using TaqMan Array Mouse Endogenous Control Assay (4,426,701, Applied Biosystems). The geometric mean of these reference genes was used to normalize gene expressions. Inter plate calibrator and none-template controls were included in all assays.

Serum analyses

Serum cholesterol, low-density lipoprotein cholesterol and very low-density lipoprotein (LDL/VLDL), glucose, triacylglycerol (TAG), total protein concentrations, uric acid, non-esterified fatty acids (NEFA; D07940, Dialab, Austria) and total antioxidant status (TAS; NX2332 Randox Total Antioxidant Status, Randox Laboratories Ltd., UK) were analysed using conventional enzymatic kits and a MaxMat PLII bioanalyser (MaxMat PL, Montpellier, France). Unless otherwise stated all kits were from MaxMat PL (Montpellier, France). Serum oxidized LDL (Ox-LDL) was quantified in duplicates according to the manufacturers' instruction with a murine ELISA-kit (E90527Mu, USCN Life Science Inc., Texas, US). Serum samples were analysed in duplicates for the following cytokines: interferon gamma (IFNγ), interleukin 10 (IL-10), interleukin 1 beta (IL-1β), interleukin 2 (IL-2), interleukin 5 (IL-5), interleukin 6 (IL-6), keratinocyte chemoattractant growth-regulated oncogene (KC-GRO), and tumour necrosis factor alpha (TNF-α). These analyses were performed according to the manufacturers' instructions using a MSD Mouse Proinflammatory panel 1 V-Plex kit (MULTI-ARRAY®, Meso Scale Discovery, Gaithersburg, MD).

Statistical analyses

All of the statistical analyses were performed using IBM SPSS Statistics for Macintosh (Release 22.0.0.0, SPSS, Inc., Chicago, IL, US). The Shapiro-Wilk's test was performed to determine the distribution of the variables and non-normally distributed variables were log-transformed before statistical analysis. Statistical difference was assessed using One-Way ANOVA with Tukeys' post hoc test or Kruskal-Wallis test. The relative gene expression was analysed with REST 2009 (V2.0.13) [29]. A value of $p < 0.05$ was considered statistically significant.

Results

The general physical health and weight gain appeared normal for all mice except one CO-fed mouse (euthanized due to weight loss) during the 13 weeks with experimental diets. The average daily feed intake (g/mice) was equal for all of the 6 diet groups (Fig. 1). Still, the mice fed CWO gained less body weight compared to the mice fed CO (Fig. 2a). Liver weights in mice fed CWO and RWO-II were lower than the liver weights in mice fed CO (Fig. 2b). These significant differences were also present for the relative liver weights. The amount of white adipose tissue was also lower in the mice fed CWO compared to the mice fed CO (1.44 g ± 0.12 vs 2.24 ± 0.2 respectively), however, not significant ($p = 0.078$). There were no apparent differences in tissue sizes between the groups for heart, kidney or spleen (Table 2). The growth curves for all of the experimental diets are presented in Fig. 2c.

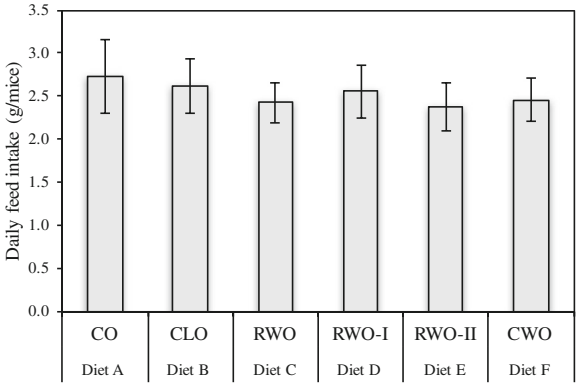

Fig. 1 Average daily feed intake (g/mice) of female apolipoprotein E-deficient mice fed high-fat diets supplemented with different oils for 13 weeks. The results are presented as mean ± SD. CO ($n = 11$), CLO ($n = 12$), RWO ($n = 12$), RWO-I ($n = 11$), RWO-II ($n = 12$), CWO ($n = 11$)

Atherosclerotic lesions

Five mice were removed from the dataset for atherosclerotic lesion analyses due to methodological artefacts or severe calcification of the abdominal aorta (from the renal arteries down to the iliac bifurcation) despite minimal lesion formation in the aortic arch region. The omitted mice were; three mice fed CLO ($n = 9$), one mouse fed RWO ($n = 11$) and one mouse fed CWO ($n = 10$). The lesion area in the aortic arch was lower in mice fed CWO compared to the lesion area of the aortic arch in mice fed CO (Fig. 3a). No significant between-group differences were observed in other regions in the aorta (abdominal part, thoracic part or even for the total aorta) (Fig. 3b-d respectively).

Serum antioxidant status, serum LDL/VLDL-cholesterol levels and serum ox-LDL levels

Serum TAS was higher in mice fed CWO and RWO-II compared to mice fed CO (Fig. 4a). TAS was also higher in mice fed RWO-I, RWO-II and CWO when compared to TAS in mice fed CLO (Fig. 4a). Serum LDL/VLDL-cholesterol levels and serum ox-LDL-cholesterol levels were lower in mice fed RWO-I, RWO-II and CWO compared to these levels in mice fed CO (Fig. 4b and c). Total cholesterol (Fig. 4d) and serum triglycerides did not differ between mice fed CO and the other diet groups; neither did serum glucose or cytokines levels (Table 2).

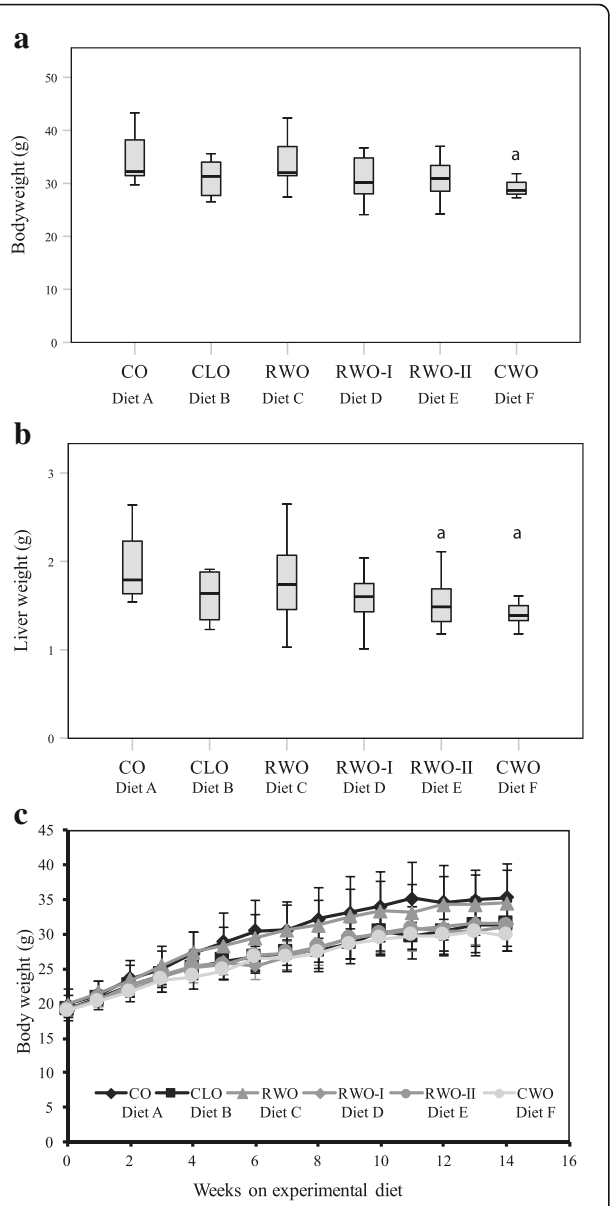

Fig. 2 Final body weight (**a**), liver weight (**b**) and growth curves (**c**) of female apolipoprotein E-deficient mice fed high-fat diets supplemented with difference oils for 13 weeks. Data are presented as box plots representing a 95% confidence interval (CI) for the median (**a** and **b**) and as bars representing mean ± SD (**c**). **a** denotes significant different from CO (diet A), $p < 0.05$. CO ($n = 11$), CLO ($n = 9$), RWO ($n = 11$), RWO-I ($n = 11$), RWO-II ($n = 12$), CWO ($n = 10$)

Gene expression

Hepatic gene expression assessments included genes involved in cholesterol and lipid metabolism, antioxidant defence and in the inflammatory response (Table 3a and b). In mice fed CLO (diet B), expression of the genes *ATP binding cassette, sub-family G member 8* (*Abcg8*) and *Scavenger receptor class B member 1* (*Sr-b1*) was enhanced (1.32 95% CI [0.64–2.61] and 1.29 95% CI [0.76–2.29]) whereas *Very low density lipoprotein receptor* (*Vldlr*) gene expression was reduced (0.75 95% CI [0.34–1.59]) compared to mice fed CO (diet A). In mice fed RWO (diet C), *Peroxisome proliferator-activated receptor-α* (*Pparα*) gene expression was enhanced (1.41 95% CI [0.67–2.66]) compared to mice fed CO. In mice fed RWO-I expression of the genes *Abcg8, Sr-b1* and *Pparα* was increased (1.43 95% CI [0.67–2.05], 1.43 95% CI [0.78–2.46] and 1.79 [0.77–3.81] respectively) compared to mice fed CO. The most dramatic changes in gene expression, when compared to mice fed CO, were observed in mice fed RWO-II and where gene expression of *Abcg8* (1.40 95% CI [0.65–2.66]), *ATP binding cassette, sub-family G member 5* (*Abcg5*) (1.28 95% CI [0.51–2.22]), *Cytochrome P450 7A1* (*Cyp7a1*) (1.95 95% CI 1.95* [0.35–8.92]), *3-hydroxy-3-methyl-glutaryl-Coenzyme A reductase* (*Hmgcr*) (1.31 [0.62–2.67]), *Sr-b1* (1.26 95% CI [0.58–2.25]), *Pparα* (1.39 95% CI [0.74–2.77]) and Peroxisome proliferator-activated receptor-γ (*Pparγ*) (1.26 95% CI [0.70–2.01]) was increased. In mice fed CWO, gene expression of *Abcg5* (1.46 95% CI [0.59–2.58]), *Sr-b1* (1.29 95% CI [0.66–2.27]) and *Pparα* (1.39 95% CI [0.60–3.09]) was enhanced compared to mice fed CO. Mice from the same group also had elevated gene expression of *Abcg8* (1.31 95% CI [0.62–2.83]), however, not significantly ($p = 0.064$). Gene expression of *Pparγ* was increased (1.25 95% CI [0.80–1.83]) in mice fed RWO compared to mice fed CLO. In mice fed RWO-I the gene expression of *Pparα* (1.57 95% CI [0.77–3.30]) was increased whereas gene expression of *Fatty acid synthase* (*Fasn*) (0.59 95% CI [0.19–2.66]) was reduced compared to mice fed CLO. Further, when comparing to mice fed CLO, it was observed that mice fed RWO-II had increased gene expression of *Cyp7a1* (2.23 95% CI 0.35–17.1]), *Vldlr* (1.52 95% CI [0.76–2.97]) and *Pparγ* (1.37 95% CI [0.87–2.06]). Finally, in mice fed CWO gene expression of *IL-6* was reduced (0.51 95% CI [0.07–3.67]) while gene expression of *Uncoupling protein 2* (*Upc2*) (1.41 95% CI [0.69–2.94]) was increased compared to mice fed CLO. Expression of all other genes were unaffected by the dietary interventions (Table 3a and b).

Discussion

The main purpose of this study was to investigate whether dietary consumption of WD supplemented with CWO (diet F) would prevent atherosclerotic development in ApoE$^{-/-}$-mice. Recently, our group demonstrated that extracts from CWO had antioxidant and anti-inflammatory activities not related to the content of LC n3-PUFA present [25]. When CWO was given as a dietary supplement to healthy volunteers, beneficial effects on CVD markers and improved anti-inflammatory effect were observed [23, 24]. Herein, WD was supplemented with 1% of CWO, or 1% RWO enriched with two different extracts from whale blubber using CO (diet A) and CLO (diet B) as control diets based on previous results [23–25].

Table 2 Serum and organ characteristics in female apolipoprotein E-deficient mice fed high-fat Western-type diets supplemented with different oils for 13 weeks

	CO Diet A	CLO Diet B	RWO Diet C	RWO-I Diet D	RWO-II Diet E	CWO Diet F
TAG (mmol/l)	2.18 ± 0.52	2.36 ± 0.61	2.35 ± 0.68	2.04 ± 0.49	2.03 ± 0.75	2.04 ± 0.74
Glucose (mmol/l)	14.5 ± 2.40	12.4 ± 1.84	15.2 ± 3.25	13.2 ± 3.87	13.6 ± 2.56	13.8 ± 2.22
Uric acid (mmol/l)	409 ± 165	399 ± 168	541 ± 211	505 ± 250	414 ± 219	354.4 ± 132
Proteins (g/l)	83.1 ± 18.2	73.3 ± 13.5	87.7 ± 16.4	85.1 ± 21.6	81.9 ± 16.7	79.3 ± 11.8
NEFA (mmol/l)	1.44 ± 0.24	1.66 ± 0.19	1.64 ± 0.25	1.56 ± 0.29	1.56 ± 0.34	1.78 ± 0.60
Spleen (g)	0.17 ± 0.05	0.17 ± 0.02	0.21 ± 0.07	0.20 ± 0.07	0.19 ± 0.05	0.17 ± 0.03
Kidney (g)	0.16 ± 0.03	0.15 ± 0.01	0.16 ± 0.02	0.16 ± 0.03	0.15 ± 0.02	0.15 ± 0.03
Heart (g)	0.15 ± 0.02	0.13 ± 0.01	0.15 ± 0.02	0.14 ± 0.02	0.14 ± 0.02	0.13 ± 0.01
Adipose (g)	2.24 ± 0.67	1.60 ± 0.58	2.03 ± 0.74	1.71 ± 0.67	2.08 ± 0.80	1.44 ± 0.38
IFNγ (pg/ml)	0.79 ± 0.97	1.26 ± 1.90	0.43 ± 0.14	0.57 ± 0.42	0.60 ± 0.51	0.45 ± 0.19
IL-10 (pg/ml)	40.7 ± 6.64	40.4 ± 17.1	40.4 ± 12.6	62.8 ± 88.6	39.1 ± 9.66	39.4 ± 10.5
IL-1β (pg/ml)	2.81 ± 1.49	2.61 ± 0.92	1.41 ± 2.68	3.29 ± 1.92	2.57 ± 2.09	3.20 ± 1.36
IL-2 (pg/ml)	1.03 ± 0.33	0.94 ± 0.40	1.13 ± 0.40	1.09 ± 0.61	1.15 ± 0.44	1.17 ± 0.27
IL-5 (pg/ml)	10.9 ± 5.54	7.82 ± 3.60	9.20 ± 4.83	9.28 ± 4.97	7.33 ± 3.19	10.2 ± 3.93
IL-6 (pg/ml)	61.1 ± 64.3	43.4 ± 28.1	50.6 ± 24.4	73.1 ± 55.5	58.4 ± 52.1	46.5 ± 28.5
KC/GRO (pg/ml)	195 ± 52.9	172 ± 43.5	168 ± 52.3	173 ± 81.2	146 ± 32.7	156 ± 63.1
TNFα (pg/ml)	25.3 ± 13.4	21.6 ± 5.90	20.1 ± 5.66	21.6 ± 10.3	24.6 ± 7.11	19.11 ± 4.80

Data are presented as mean ± SD. CO ($n = 11$), CLO ($n = 9$), RWO ($n = 11$), RWO-I ($n = 11$), RWO-II ($n = 12$), CWO ($n = 10$). None of the values for any group in this table are significantly different from values in any other group

This study indicates that a physiologically obtainable dietary supplementation with CWO prevents WD-induced atherogenesis. Mice fed a diet supplemented with 1% CWO for 13 weeks had significantly lower development of atherosclerotic lesions in the aortic arch compared to mice fed WD supplemented with 1% CO. There was, however, no differences in lesion formation in less lesion-prone parts of the aorta, such as the thoracic aorta, abdominal aorta or the total aorta. It was also evident that serum levels of LDL/VLDL-cholesterol and ox-LDL-cholesterol were markedly reduced, whereas serum TAS was significantly enhanced in CWO-fed mice compared to mice fed CO or CLO. This is in accordance with our recent demonstration that extracts from whale blubber have in vitro antioxidative effects [25]. A similar anti-atherosclerotic effect has previously been reported in ApoE$^{-/-}$-mice fed seal oil [27]. It is, however, worthy to note that the seal oil was given in combination with extra virgin olive oil known to contain protective antioxidants. Antioxidants prevent lipid peroxidation and oxidative damage [30]. Indeed, the results herein suggest that CWO does not need a further addition of antioxidants, something which was shown by the high antioxidative capacity observed in our in vitro study [25]. Together, the increased TAS levels and lower ox-LDL-cholesterol levels explain the observed reduction of atherosclerotic lesions in the aortic arch observed in the CWO-fed mice. In addition, the final body weights were lower in the CWO-fed mice even though the feed intake was equal. At the same time, no differences in serum TAS levels or final body weights were observed between the CLO- and the CO-fed mice.

Although cholesterol is crucial for all mammalian cells, it is well known that increased circulating levels of LDL-cholesterol is a major risk factor for atherosclerotic CVD. In recent years much effort has been put into reducing circulation cholesterol levels in high-risk patients [31]. The liver is the major site for cholesterol synthesis, and de novo homeostasis is regulated by intestinal absorption and faecal and biliary excretion [32]. To shed light on the mechanisms involved in the observed LDL/VLDL-lowering effect of CWO, several receptors and enzymes involved in the cholesterol metabolism were investigated by means of gene expression analysis. In our study, expression of the hepatic genes *Abcg5*, *Abcg8* ($p = 0.064$), *Sr-b1* and *Pparα* were moderately up-regulated in mice fed CWO compared to mice fed CO. All these genes encode for proteins that are important for cholesterol homeostasis. The liver receptor SR-B1 is involved in the selective uptake of cholesteryl esters (CE) from high density lipoproteins (HDL) both in humans and mice [33]. The hepatic expression of *Sr-b1* has been shown to play an atheroprotective role associated with its impact on circulation cholesterol levels [34]. CE is further converted into bile by the obligate heterodimer ABCG5 and ABCG8 located in the hepatocytes [35].

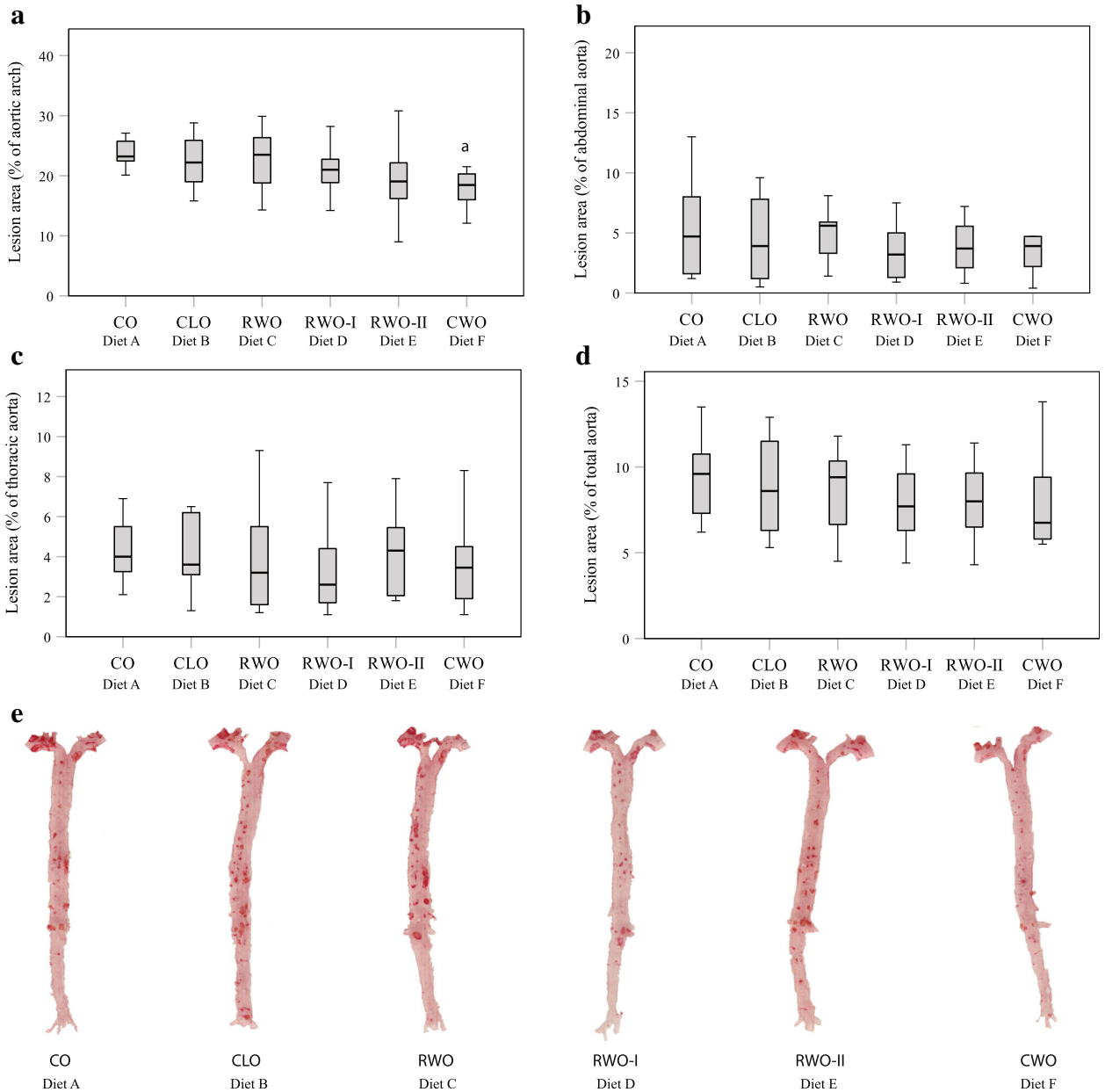

Fig. 3 Box plot of atherosclerotic plaque burden expressed as aortic area percentage covered by lipid Oil Red O staining in female apolipoprotein E-deficient mice fed high-fat diets supplemented with different oils for 13 weeks (**a**-**d**). Data are presented as a 95% confidence interval (CI) for the median, and **a** denotes significant difference from mice fed CO (diet A) with $p < 0.05$. CO ($n = 11$), CLO ($n = 9$), RWO ($n = 11$), RWO-I ($n = 11$), RWO-II ($n = 12$), CWO ($n = 10$). (**e**) shows a representative aorta from each group stained with Oil Red O Staining

Overexpression of *Abcg5*/*Abcg8* has been shown to increase biliary cholesterol excretion, together with reduced levels of liver inflammatory markers [36]. This effect was lower in mice fed CLO as only expression of *Abcg8* and *Sr-b1* were increased compared to the CO-control mice. This corresponds well with the increased expression of *Abcg5* together with reduced circulating levels of both LDL/VLDL-cholesterol and ox-LDL-cholesterol observed in CWO-fed mice. At the same time, these mice displayed no changes in inflammatory markers as assessed by hepatic gene expression and serum protein levels. PPARα is a nuclear receptor involved in transport, uptake, oxidation and reduction of FA and the triglyceride synthesis [37], and may also be involved in the observed effects. Natural ligands for PPARα include n3-PUFA and it is possible that the diets containing the marine oils may activate PPARα and contribute to the beneficial effects observed in this study.

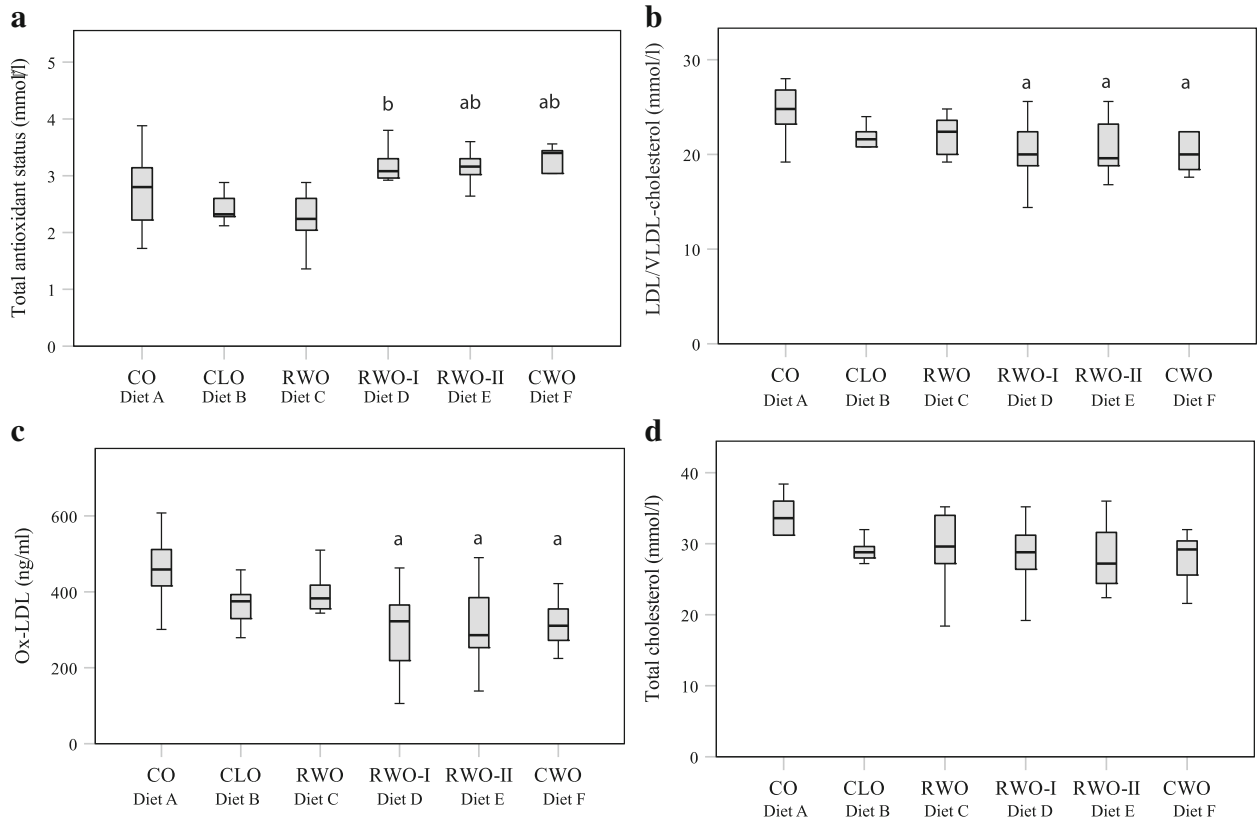

Fig. 4 Box plot of serum total antioxidant status (**a**), LDL/VLDL-cholesterol levels (**b**), ox-LDL-cholesterol levels (**c**) and total cholesterol (**d**) in female apolipoprotein E-deficient mice fed high-fat diets supplemented with different oils for 13 weeks. Data are presented with 95% confidence interval (CI), and **a** denotes significant different from mice fed CO (diet A) whereas **b** denotes significant difference from mice fed CLO (diet B) with $p < 0.05$. CO ($n = 11$), CLO ($n = 9$), RWO ($n = 11$), RWO-I ($n = 11$), RWO-II ($n = 12$), CWO ($n = 10$)

We were further interested in whether the beneficial effects from CWO might be obtained via reconstitution of the extract(s) with RWO. Hence, two different extracts were prepared and reconstituted with RWO and the mice received three addition diets supplemented with (i) RWO (diet C), (ii) RWO + extract I (RWO-I, diet D) and (iii) RWO + extract II (RWO-II, diet E). This would indicate whether the anti-inflammatory activity was associated with 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). Indeed, compared to corn oil both RWO-I and RWO-II also reduced serum LDL/VLDL-cholesterol and ox-LDL-cholesterol concentration, whereas serum total antioxidant status was increased. Mice fed RWO-I had increased hepatic expression of *Abcg8*, *Sr-b1* and *Pparα*, and mice fed RWO-II had even more affected genes as the hepatic expression of *Abcg5*, *Abcg8*, *Cyp7al*, *Hmgcr*, *Sr-b1*, *Pparα* and *Ppary*, were all increased. The mice fed RWO-II also had increased expression of *Cyp7al*, *Vldlr* and *Ppary* compared to the CLO-fed mice. However, no changes were observed for atherosclerosis (not significantly reduced) or inflammation markers. This indicated that the extracts affected the same metabolic processes, however, the phenotypic effects were moderate compared to the effects observed in CWO-fed mice.

Due to the lower content of EPA and DHA in whale blubber compared to fish oils, we expected, and observed, unaltered serum TAG levels. Unaltered serum TAG levels have been observed in several other studies where $ApoE^{-/-}$-mice were given comparable amounts of EPA, DPA and DHA [38–40]. Efficient reduction of serum TAG levels requires EPA, DPA and DHA supplementation in pharmaceutical doses which may be difficult to obtain through diet [41]. Whale blubber oil is known to contain large amounts of DPA compared to fish oil, and some studies have indicated that DPA may be the most important LC n3-PUFA when it comes to protection of CVD [42, 43]. Taken together these findings may imply that dietary CWO promotes cholesterol clearance from the circulation, increased clearance of cholesterol in the liver elevated TAS levels, lowered oxidation of LDL-cholesterol, all conducting to mitigate atherosclerosis.

Table 3 Hepatic gene expression in female apolipoprotein E-deficient mice fed high-fat Western-type diets supplemented with different oils for 13 weeks

a)	CLO Diet B [95% CI]	RWO Diet C [95% CI]	RWO-I Diet D [95% CI]	RWO-II Diet E [95% CI]	CWO Diet F [95% CI]
Tnfa	1.03 [0.24–2.59]	0.76 [0.16–2.35]	0.79 [0.11–5.21]	0.76 [0.14–2.38]	1.18 [0.16–13.9]
Mcp1	1.01 [0.37–2.70]	0.94 [0.35–2.59]	0.79 [0.11–3.15]	0.80 [0.26–2.78]	0.92 [0.23–3.27]
Il-6	1.39 [0.42–5.14]	0.87 [0.26–2.52]	1.09 [0.26–3.07]	1.18 [0.29–4.20]	0.79 [0.11–4.22]
Icam1	1.11 [0.70–1.704]	1.08 [0.65–0.21]	1.09 [0.462–2.46]	0.74 [0.02–1.87]	1.18 [0.52–2.25]
Vcam1	0.85 [0.47–1.38]	0.86 [0.55–1.31]	0.88 [0.57–1.40]	0.89 [0.50–1.29]	0.85* [0.50–1.39]
Pon2	0.96 [0.76–1.22]	0.99 [0.76–1.29]	0.98 [0.83–1.33]	0.96 [0.81–1.12]	1.00 [0.73–1.25]
Nfe212	0.98 [0.62–1.47]	1.01 [0.64–2.04]	0.93 [0.57–1.52]	1.01 [0.65–1.48]	1.06 [0.61–1.76]
Upc2	0.96 [0.43–1.50]	1.03 [0.49–1.58]	1.03 [0.36–2.33]	1.11 [0.45–2.01]	1.35 [0.49–3.02]
Abcg5	1.15 [0.45–1.90]	1.06 [0.40–1.71]	1.08 [0.31–2.42]	1.28* [0.51–2.22]	1.46* [0.59–2.58]
Abcg8	1.32* [0.64–2.61]	1.12 [0.56–2.16]	1.43* [0.67–2.05]	1.40* [0.65–2.66]	1.31 [0.62–2.83]
Acat2	1.00 [0.64–1-66]	1.02 [0.50–2.22]	1.02 [0.57–1.83]	1.09 [0.48–2.30]	0.91 [0.50–1.52]
Cyp7a1	0.88 [0.11–4.85]	1.19 [0.34–4.47]	1.48 [0.23–7.65]	1.95* [0.35–8.92]	1.19 [0.37–6.34]
Hmgcr	1.16 [0.62–2.12]	1.11 [0.60–2.22]	1.12 [0.55–2.38]	1.31* [0.62–2.67]	1.04 [0.49–2.40]
Ldlr	1.13 [0.57–2.55]	1.14 [0.61–2.09]	1.27 [0.57–2.67]	1.19 [0.61–2.38]	1.03 [0.40–2.55]
Sr-b1	1.29* [0.76–2.29]	1.23 [0.67–2.30]	1.43* [0.78–2.46]	1.26* [0.58–2.25]	1.29* [0.66–2.27]
Vldlr	0.75* [0.34–1.59]	0.92 [0.46–1.75]	0.87 [0.23–2.05]	1.14 [0.58–2.11]	0.79 [0.30–1.61]
Ppara	1.14 [0.53–2.22]	1.41* [0.67–2.66]	1.79* [0.77–3.81]	1.39* [0.74–2.77]	1.39* [0.60–3.09]
Pparγ	0.92 [0.54–1.39]	1.15 [0.67–1.71]	1.16 [0.52–2.21]	1.26* [0.70–2.01]	1.12 [0.47–9.86]
Fasn	1.21 [0.35–5.39]	1.01 [0.20–4.31]	0.72 [0.22–3.60]	0.94 [0.21–4.88]	0.81 [0.20–3.74]
b)	CO Diet A [95% CI]	RWO Diet C [95% CI]	RWO-I Diet D [95% CI]	RWO-II Diet E [95% CI]	CWO Diet F [95% CI]
Tnfa	0.97 [0.39–4.10]	0.74 [0.36–1.69]	0.77 [0.15–4.35]	0.74 [0.29–1.69]	1.15 [0.25–11.0]
Mcp1	0.99 [0.37–2.70]	0.93 [0.44–2.15]	0.78 [0.09–3.45]	0.79 [0.27–2.29]	0.92 [0.22–2.73]
Il-6	0.72 [0.20–2.43]	0.63 [0.15–2.25]	0.79 [0.16–2.62]	0.85 [0.16–3.70]	0.51* [0.07–3.67]
Icam1	0.90 [0.59–1.44]	0.98 [0.69–1.90]	0.98 [0.42–2.25]	0.67 [0.02–1.59]	1.06 [0.45–1.89]
Vcam1	1.18 [0.72–2.14]	1.01 [0.57–2.01]	1.03 [0.57–1.98]	1.05 [0.50–1.98]	1.00 [0.51–2.09]
Pon2	1.05 [0.82–1.32]	1.04 [0.77–1.39]	1.02 [0.79–1.39]	1.00 [0.78–1.26]	1.05 [0.77–1.39]
Nfe212	1.03 [0.68–1.62]	1.04 [0.74–2.14]	0.95 [0.55–1.45]	1.03 [0.73–1.40]	1.09 [0.70–1.75]
Upc2	1.04 [0.67–2.35]	1.07 [0.71–1.54]	1.07 [0.59–2.14]	1.15 [0.63–1.98]	1.41* [0.69–2.94]
Abcg5	0.87 [0.53–2.21]	0.93 [0.57–1.47]	0.94 [0.24–2.07]	1.12 [0.64–1.89]	1.27 [0.63–2.22]
Abcg8	0.76* [0.38–1.58]	0.85 [0.46–1.71]	1.09 [0.58–2.14]	1.06 [0.53–2.06]	0.99 [0.51–2.14]
Acat2	0.99 [0.60–1.55]	1.01 [0.48–2.13]	1.01 [0.56–1.94]	1.09 [0.45–2.46]	0.91 [0.49–1.55]
Cyp7a1	1.14 [0.21–8.90]	1.36 [0.35–9.16]	1.69 [0.23–14.4]	2.23* [0.35–17.1]	1.36 [0.37–10.5]
Hmgcr	0.86 [0.47–1.62]	0.96 [0.62–1.63]	0.97 [0.58–1.72]	1.13 [0.60–2.18]	0.90 [0.50–2.00]
Ldlr	0.89 [0.39–1.76]	1.01 [0.49–1.78]	1.12 [0.30–2.77]	1.05 [0.49–1.98]	0.91 [0.36–2.09]
Sr-b1	0.77* [0.44–1.32]	0.95 [0.54–1.61]	1.10 [0.65–1.74]	0.97 [0.49–1.57]	0.99 [0.54–1.59]
Vldlr	1.34* [0.63–2.99]	1.23 [0.62–2.37]	1.17 [0.30–2.77]	1.52* [0.76–2.97]	1.05 [0.39–2.46]
Ppara	0.88 [0.45–1.89]	1.23 [0.53–2.24]	1.57* [0.77–3.30]	1.21 [0.76–2.97]	1.22 [0.61–2.91]
Pparγ	1.09 [0.72–1.84]	1.25* [0.80–1.83]	1.26 [0.56–2.27]	1.37* [0.87–2.06]	1.22 [0.59–10.6]
Fasn	0.83 [0.19–2.85]	0.84 [0.14–2.99]	0.59* [0.19–2.66]	0.78 [0.18–3.34]	0.66 [0.15–1.27]

Relative comparison of mice fed CO to the other diets group (**a**), and relative comparison of mice fed CLO to the other diets group (**b**). 95% confidence interval [95% CI]. The relative gene expression was analysed with REST 2009 and *denotes significant difference ($p < 0.05$). CO ($n = 11$), CLO ($n = 9$), RWO ($n = 11$), RWO-I ($n = 11$), RWO-II ($n = 12$), CWO ($n = 10$)

Conclusion

In the present study, we observed that mice fed WD with 1% CWO had reduced formation of atherosclerotic lesions in the aortic arch compared to mice fed WD with 1% CO. In addition, CWO-fed mice had reduced serum LDL/VLDL-cholesterol, ox-LDL-cholesterol and body weight and increased serum total antioxidant status compared to CO-fed mice. Our study adds novel insight into the putative protective mechanisms of dietary CWO supplementation in CVD, including activation of endogenous antioxidant responses, inhibition of LDL oxidation, attenuation of the diet-induced hypercholesterolemia and reduced aortic atherogenesis. These results may inform future dietary recommendations to reduce CVD and promote public health.

Additional file

Additional file 1: Table S1. Predesigned TaqMan® Gene Expression assays # Reference genes used to normalize the results. (DOCX 14 kb)

Abbreviations

ABCG5: ATP binding cassette, sub-family G member 5; ABCG8: ATP binding cassette, sub-family G member 8; ACAT2: Acetyl-Coenzyme A acetyltransferase 2; ApoE$^{-/-}$: Apolipoprotein E-deficient; 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; DPA: Docosapentaenoic acids; EPA: Eicosapentaenoic acid; FA: Fatty acids; FASN: Fatty acid synthase; HDL: High density lipoproteins; HMGCR: 3-hydroxy-3-methyl-glutaryl-Coenzyme A reductase; HPRT1: Hypoxanthine-guanine phosphoribosyltransferase 1; ICAM1: Intercellular adhesion molecule 1; IFNγ: Interferon gamma; IL-10: Interleukin 10; IL-1β: Interleukin 1 beta; IL-2: Interleukin 2; IL-5: Interleukin 5; IL-6: Interleukin 6; KC-GRO: Keratinocyte chemoattractant growth-regulated oncogene; LC n3-PUFA: Long chain n3-polyunsaturated fatty acid; LDL: Low density lipoprotein; LDLR: LDL-receptor; MCP-1: Monocyte chemotactic protein 1; n3-PUFA: n3-polyunsaturated fatty acid; NEFA: Non-esterified fatty acids; NFE212: Nuclear factor erythroid 2-related factor; Ox-LDL: Oxidized low density lipoprotein; PON2: Paroxynase 2; PPARα: Peroxisome proliferator-activated receptor-α; PPARγ: Peroxisome proliferator-activated receptor-γ; PUFA: Polyunsaturated fatty acid; RWO: Refined whale oil; RWO-I: Refined whale oil + extract I; RWO-II: Refined whale oil + extract II; SR-B1: Scavenger receptor class B member 1; TAG: Triacylglycerol; TAS: Serum total antioxidant status; TBP: TATA-Box Binding Protein; TNFα: Tumour necrosis factor alpha; UCP2: Uncoupling protein 2; VCAM1: Vascular adhesion molecule 1; VLDL: Very low density lipoprotein; VLDLR: Very low density lipoprotein receptor; WD: Western type diet

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Authors' contributions

KEE, BØ, EOE and SKS conceived and designed the study, MJW, KEE and SKS performed the experiments. MJW and KEE analyzed the data, and MJW and KEE wrote the paper. All authors contributed to the finalization of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Norwegian Animal Research Authority approved the study (approval number 3828). All experiments were performed following Federation for Laboratory Animal Science Association recommendations and according to the Norwegian legislation on the care and use of experimental animals.

Competing interests

The authors declare no competing interests, neither financial nor non-financial.

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Paper III

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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

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Abstract

Background: It is now increasingly recognized that the beneficial effects of seafood consumption is not limited to lipids and fatty acid, but that the protein part, i.e., peptides and amino acids, together with vitamins and even unknown bioactive constituents also are important for disease prevention. This study was designed to evaluate the putative anti-atherogenic effects of different protein sources (a lean seafood and a nonseafood) in apolipoprotein E-deficient (apoE$^{-/-}$) mice.

Methods: Twenty-four 5-week-old female apoE$^{-/-}$ mice were fed Western type diets containing chicken or a combination of cod and scallops as dietary protein sources for 13 weeks. Atherosclerotic plaque burden, weight, serum levels of leptin, glucose and LDL cholesterol as well as gene expressions from liver and heart were evaluated. Statistical analyses were performed using SPSS. Differences between the variables were evaluated using independent *t*-test or Mann–Whitney *U* test for normally and non-normally distributed variables, respectively. Normality was defined by the Shapiro-Wilk test.

Results: The mice fed cod-scallop had a 24 % ($p < 0.05$) reduced total aorta atherosclerotic plaque burden compared to the chicken fed group, whereas the reduction in plaque in the less lesion prone thoracic and abdominal parts of the descending aorta were 46 % ($p < 0.05$) and 56 % ($p < 0.05$), respectively. In addition, mice fed cod-scallop gained less weight, and had lower serum levels of leptin, glucose and LDL cholesterol, compared to those fed chicken. Analysis of expression of the genes from liver and heart showed that hepatic endogenous antioxidant paraoxonase 2 (*Pon2* gene) and the vascular cell adhesion molecule VCAM-1 (*Vcam1* gene) were down regulated in mice fed cod-scallop compared to mice fed chicken.

Conclusion: The present study revealed a metabolic beneficial effect of lean seafood compared to chicken, as atherosclerotic plaque burden, serum glucose, leptin and LDL cholesterol levels were reduced in mice fed cod-scallop.

Keywords: Apolipoprotein E-deficient mice, Atherosclerosis, Plaque burden, Dietary intake, cod, Scallop, Lean seafood, Gene expression

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Background

In spite of tremendous developments in preventive and acute treatment of cardiovascular diseases (CVD), CVD is still the largest cause of morbidity and pre-mature mortality worldwide [1]. Important factors that contribute to this is the increased consumption of energy dense and processed foods together with a decline in physical activity [2]. Atherosclerosis is a multifactorial inflammatory condition affecting the arteries by plaque formation and subsequent narrowing of the lumen. Diet has a major impact on the atherosclerotic disease initiation and progression and nutritional intervention is considered an effective and safe approach to health maintenance and CVD prevention [3]. Indeed, a change in nutritional pattern has been estimated to reduce CVD-related deaths by up to 60 % [4].

A diet high in fish and seafood has long been acknowledged to reduce risk of CVD [5–7], mainly due to the effects of the omega-3 fatty acids EPA (eicosapentaenoic acid, 20:5n3) and DHA (docosahexaenoic acid, 22:6n3). It has, however, become increasingly evident that the beneficial effects of seafood consumption are not limited to lipids and fatty acids. Proteins, peptides and amino acids together with vitamins and even unknown bioactive constituents may also be important for disease prevention by controlling circulating levels of cholesterol, lipoproteins and triglycerides, increasing endogenous antioxidants and lowering blood pressure [8–13]. The exact mechanisms behind these effects, however, remain unknown.

Seafood is generally recognized as a rich source of taurine [14] which might have a positive impact on CVD [15, 16]. Recently, it was shown that male C57BL/6J mice fed a high-fat, high-sugar diet containing scallop, endogenously high in taurine and glycine, as protein source, had improved plasma lipid profile, as compared to mice fed high fat, high sugar diets with chicken fillet or cod as the protein sources [17]. Thus, the present study was undertaken to elucidate whether intake of a Western-type diet with a mixture of cod and scallop protein, as compared to chicken fillet, would prevent development of CVD in the atherosclerosis-prone model Apolipoprotein E-deficient (apoE$^{-/-}$) mice.

A substantial metabolic beneficial effect of the marine protein sources was documented, as atherosclerotic plaque burden, serum glucose, leptin and LDL cholesterol levels were reduced in mice fed cod-scallop.

Methods

Diets

The mice were fed a Western type diet (Research diet D12079B, 17 energy percent protein, 43 energy percent carbohydrates and 40 energy percent fat, Table 1) in which the standard protein source casein was fully exchanged with either chicken fillet or a mixture of cod fillet and scallop muscle (1:1 on nitrogen basis). The amino acid methionine was added to ensure adequate metabolic balance. The lipid, protein and energy contents were 20–21g/100g, 15.9–16.9g/100g and 20.2–21.0kJ/g, respectively, and the cholesterol contents were 2.0 and 1.9g/kg for the chicken and cod-scallop diet (Table 2). The amino acid compositions were similar between the two diets (Table 3) with a few exceptions. Histidine was more abundant in chicken than in the cod-scallop diet, while the cod-scallop diet had almost twice the amount of glycine compared to chicken diet (10.1 and 5.7mg/g, respectively). Further, the cod-scallop diet had a taurine content 5.3mg/g, whereas chicken diet was almost devoid of taurine. The percentage distribution of fatty acids (Table 4) where similar between the diets with the exceptions of EPA and DHA which were higher in cod-scallop diet compared to chicken diet and arachidonic acid which was higher in the chicken diet compared to the cod-scallop diet.

Table 1 Composition of experimental diets (g/kg)

	Dietary treatments	
	Chicken	Cod-scallop
Chicken fillet	189.8	0
Cod fillet	0	92.9
Scallop	0	102.9
L-methionine	3	3
Corn starch	55.6	49.7
Maltodextrine	100	100
Sucrose	341	341
Cellulose	50	50
Milk fat (anhydrous)	200	200
Corn oil	10	10
Mineral mix S1001	35	35
Calcium carbonate	4	4
Vitamin mix V1001	10	10
Choline bitartrate	2	2
Cholesterol	1.1	1.1
Ethoxyquin	0.04	0.04

Table 2 Analyzed composition of the experimental diets (g/kg)

	Dietary treatments	
	Chicken	Cod-scallop
Crude protein (N*6.25)	169	159
Fat	210	200
Cholesterol	2.0	1.9
Gross energy (kJ/g)	21	20.2

Table 3 Amino acid composition of the experimental diets (mg/g)

	Dietary treatments	
	Chicken	Cod-scallop
Hydroxyproline	0.2	0.2
Histidine	4.5	2.2
Taurine	0.1	5.3
Serine	5.8	5.7
Arginine	8.1	8.9
Glycine	5.7	10.1
Asparagine + aspartic acid	15.4	15.2
Glutamine + glutamic acid	23.6	22.2
Threonine	6.6	5.7
Alanine	9.0	8.3
Proline	5.2	4.1
Lysine	14.7	13.2
Tyrosine	3.5	3.0
Methionine	6.3	6.3
Valine	7.6	6.1
Isoleucine	7.3	6.1
Leucine	12.0	10.6
Phenylalanine	5.5	4.7
Cysteine	1.9	2.1
Tryptophan	1.62	1.23
SAA	144.52	135.93
EAA[1]	66.12	56.13
BCAA[2]	26.9	22.8

SAA sum amino acids; [1]Sum of essential amino acids (histidine, isoleucine, leucine, methionine, lysine, valine, tryptophane, threonine, phenylalanine). [2]Sum of branched-chain amino acids (isoleucine, leucine and valine)

Table 4 Fatty acid (FA) composition as percentage of total FA and mg/g of the experimental diets

	Dietary treatments			
	Chicken		Cod-scallop	
FA	%	mg/g	%	mg/g
<14:00	8.4	15.7	8.2	14.3
14:00	10.3	19.1	10.6	18.4
16:00	28	52.0	28.2	48.8
16:1n7	1.5	2.83	1.5	2.56
18:00	10.7	20.0	10.6	18.4
18:1n9	23.6	43.9	22.6	39.1
18:1n7	1.0	1.8	1	1.7
18:2n6	5.4	10.0	4.2	7.27
18:3n3	0.7	1.2	0.6	0.97
20:1n9	0.1	0.2	0.1	0.17
20:4n6	0.2	0.4	0.1	0.24
20:5n3	0.1	0.1	0.5	0.79
22:5n3	0.1	0.23	0.1	0.21
22:6n3	0.1	0.17	0.7	1.25
SFA	59.2	110	59.6	103
MUFA	29.7	55.2	28.8	49.9
n-3	1.0	1.76	1.9	3.22
n-6	5.6	10.4	4.3	7.51
n6/n3	5.6	5.9	2.3	2.3
Sum identified	96.1	179	95.3	165
Sum nonidentified	3.9	7.29	4.7	8.08

Protein, lipid and energy content of feed

The feed was analyzed for crude protein, total fat and gross energy content. Nitrogen content was determined after combustion using the Dumas method (Leco FP-528, ISO 34/SC 5, ON, Canada) and crude protein content was calculated as nitrogen content multiplied by 6.25. Gross energy was determined using an automatic adiabatic calorimeter (Model 1241; Parr Instruments, Moline, IL, USA). Total fat was determined gravimetrically by extraction with organic solvents before and after acidic hydrolysis, as described previously [17]. The amino acid composition was determined after acidic hydrolysis by the Acquity UPLC system (Waters, USA), the tryptophan content was determined after basic hydrolysis on a HPLC system (Shimadzu, USA) as described previously in details [17]. Total cysteine in the samples was determined after oxidation of cysteine/cystine with 9:1 performic acid (88 %): H_2O_2 (30 %) (v/v) to yield cysteic acid. Total cysteine analysis was performed by the Norwegian Institute of Food, Fishery and Aquaculture (Bergen, Norway). Total cholesterol was determined on a Thermo Trace 2000 GC equipped with a flame ionization detector (FID) operated under conditions described elsewhere [18]. Prior to injection on the GLC, the samples had been added internal standard α-5 cholestan, saponified in 0.5M NaOH/MeOH at 80 °C for 15 min, hexan-extracted, dried under N_2 at 60 °C, and resuspended in hexan.

Animals and housing

Apolipoprotein E-deficient mice fed a Western type, high fat diet, were used as model system. Such apoE$^{-/-}$ mice develop atherosclerosis spontaneously with similar characteristics as those seen in humans [19, 20] and are extensively used to study dietary effects on atherosclerosis. Twenty-four female apoE$^{-/-}$ mice (B6.129P2-*Apoe*^{tm1Unc}N11), 5-weeks-old, were obtained from Taconic, Denmark. After one week of acclimation, the mice were ear marked and randomly assigned to the experimental groups (n = 12/group) with equal number of cages per treatment (n = 4 cages/diet). All mice were housed in individually ventilated cages in the same room at 21 °C and 55 % relative humidity, on a 12 h

day/night cycle (light: 0600 to 1800 h) in a conventional laboratory animal unit. The mice had unrestricted access to feed that was distributed in wire bar lids with food hoppers for 13 weeks. Cages and bedding were changed once per week. After 13 weeks of feeding, the mice were feed-deprived for 3 h before they were euthanized by carbon dioxide inhalation. Blood was drawn by cardiac puncture and serum separated and frozen at −80 °C. Tissues were dissected out, weighted, snap frozen and kept at −80 °C. The study was approved by the Norwegian Animal Research Authority (Approval number 3277) and performed following Federation of European Laboratory Animal Science Associations recommendations and according to the Norwegian legislation on the care and use of experimental animals. The mice were pathogen free, stated by a health certificate. Adverse events were not observed.

Analysis of atherosclerosis

After blood removal by cardiac puncture, the mouse was immediately perfused through the left ventricle with sterile saline (0.9 %) for approximately 5 min until no residual blood was apparent in the circulation. The entire aorta from the proximal ascending aorta to the bifurcation of the iliac arteries was dissected and cleaned in situ from periadventitial fat. The aorta was opened longitudinally, fixed and prepared by lipid Oil Red O-staining and subjected to whole mount *en face* evaluation as previously described [21]. Aorta images were made by scanning the objective on a high resolution scanner and lesion areas were evaluated using ImageJ software [22]. The extent of atherosclerotic burden was reported as percentage of the total area of an artery, or artery region, i.e., the aortic arch, the thoracoabdominal part of the descending aorta (thoracic area), and the infrarenal part down to the iliac bifurcation (abdominal area), occupied by atherosclerotic lesions.

RNA-isolation

Total RNA was isolated from liver and heart tissue. Immediately after perfusion, tissue was dried, weighted and frozen in liquid nitrogen and kept at −80 °C until analysis. Tissue (30mg to 90mg) was cut in liquid nitrogen and homogenized in 1 ml Trizol (Invitrogen) using a bead miller (Precellys 24, Bertin Technologies). After centrifuging the homogenate for 10 min at 12000g and 4 °C, the supernatant was mixed generously with 0.2 volumes chloroform, incubated 30 min on ice and centrifuged for 20 min at 12000g and 4 °C. The aqueous phase was mixed with 0.5 volumes fresh isopropanol and incubated on ice for a minimum of 2 h before 30 min centrifugation at full speed (21000g) and 4 °C. The remaining pellet was washed with ice-cold 80 % ethanol, centrifuged 5 min at 21000g and 4 °C, dried and dissolved in RNA storage solution (Ambion) and stored at −80 °C. RNA concentration was determined using a Qubit 1.0 fluorometer (Life technologies) and the RNA integrity was evaluated using a 2100 Bioanalyzer Instrument (Agilent Technologies). The relative integrity numbers of the isolates were between 8.1 and 9.4.

Reverse transcription and quantitative real-time PCR

Reverse transcription was performed in triplicate using the high capacity cDNA RT kit (Applied Biosystems) with 50ng RNA in 20μl reactions. Four μl cDNA (12.5ng of reverse transcribed RNA) per 20μl reaction was analyzed by quantitative RT-PCR using predesigned TaqMan® Gene Expression assays (Additional file 1: Table S1) and TaqMan Fast Universal PCR Master Mix (Applied Biosystems) in 96-well plates with an ABI Prism 7500 Fast Cycler (Applied Biosystems). The amplification profile was 95 °C for 20s followed by 40 cycles of 95 °C for 3s and 60 °C for 30s. Endogenous reference genes were selected using the TaqMan Array Mouse Endogenous Control assays. From this panel of 32 housekeeping genes, the two most stably expressed control genes (*Hprt* and *Tbp*) were selected, and the geometric mean of the two was used to normalize gene expressions. The calculations were made using the relative expression software tool [23]. All assays included no-template-controls and an inter plate calibrator.

Serum cholesterol, triglyceride, glucose and total protein concentrations

The serum cholesterol, LDL cholesterol, triglyceride, glucose and total protein concentrations were determined by conventional enzymatic kits using a MaxMat bioanalyzer (MaxMat PL II, Montpellier, France).

Hepatic fat and fatty acid analysis

Total hepatic fat was extracted using dichloromethane and methanol, and determined gravimetrically [24]. The fatty acid composition of the liver samples was determined as previously described [25].

Serum cytokines, chemokines and hormones

Serum samples were analyzed in duplicates according to the manufacturers' instructions using a MSD Mouse Proinflammatory panel 1 V-Plex kit (MULTI-ARRAY®, Meso Scale Discovery, Gaithersburg, MD) and a Bio-Plex Pro Mouse Diabetic Assay (MAGPIX, Bio-Rad, Copenhagen, Denmark). Serum cytokines, chemokines and hormones analyzed were IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/GRO, and TNF-α using MSD and glucagon, insulin, leptin, monocyte chemotactic protein-1 (MCP-1), and regulated upon activation, normal T cell expressed and secreted (RANTES) using Bio-Plex (Bio-Rad).

Statistical analysis

The results are presented as mean ± standard error of the mean. SPSS 19.0 (SPSS Inc., Chicago, IL) was used to perform statistical analysis of the data. The Shapiro-Wilk test was performed to determine the distribution of the variables and non-normal distributions were log-transformed before statistical analysis with an independent *t*-Test. Variables that were non-normally distributed after log-transformation were analyzed with Mann–Whitney *U* test. Bivariate analyses of correlation between parameters were assessed by Pearson's correlation coefficient. Differences were considered significant when $p < 0.05$.

Results

General outcomes and mice growth

The mice appeared in good physical shape and gained weight throughout the experimental period. The exceptions were two mice in the cod-scallop group that were euthanized due to weight loss during the first weeks of the study. These two mice were small at arrival and should probably have been removed prior to the start of the study. Although the average daily energy intake was similar in both groups ($p = 0.88$), the mice fed chicken protein had gained significantly more weight than mice fed cod-scallop protein (21.4g vs 16.6g, $p < 0.05$) at the end of the experiment (Fig. 1). The visceral adipose tissue, represented by the epididymal and the perirenal fat, weight was higher (3.15g vs 2.45g, $p < 0.05$) in the chicken fed group compared to the cod-scallop fed group (Fig. 2a), but when calculated as relative to body weight, no significant difference was observed (Fig. 2b). Heart weight was not different between cod-scallop and chicken fed mice.

Atherosclerotic lesions

The atherosclerotic lesions were predominantly distributed in the aortic arch and in the areas surrounding the branching points of the major arteries (Fig. 3). The plaque burdens in the aorta thoracic, abdominal as well as total area were reduced by 46 %, 56 % and 24 % ($p = 0.004$, 0.003 and 0.044), respectively in mice fed cod-scallop compared to mice fed chicken. Also in the most plaque prone area, the aortic arch, the cod-scallop fed mice had lower plaque burden, however, the difference did not reach statistical significance ($p = 0.09$).

Hepatic fat content and fatty acid composition

The hepatic fat content extracted with dichloromethane/methanol was higher in chicken fed mice compared to cod-scallop fed mice (430mg/g and 330mg/g, respectively, $p < 0.05$) (Table 5). The concentration of hepatic fatty acids was borderline ($p = 0.054$) higher in chicken fed mice compared to cod-scallop fed mice, 194 versus 152mg/g liver, respectively. When estimating total liver

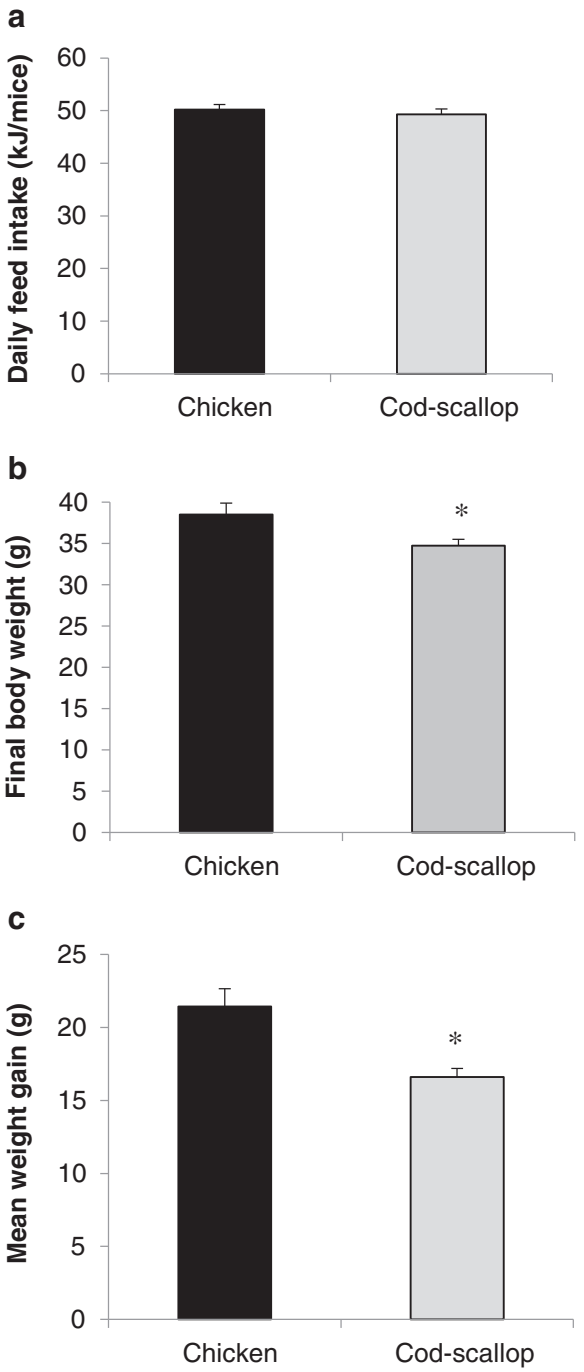

Fig. 1 Feed intake and weight. (**a**) average daily feed intake (kJ/mice); (**b**) final body weight; (**c**) body weight gain (g) of female apolipoprotein E-deficient mice fed diets containing different protein sources for 13 weeks. Data are mean ± SEM. Average daily feed intake was analyzed using the non-parametric Mann–Whitney *U* test, while final body weight and body weight gain were analyzed using the independent samples *t*-Test. * denotes significant difference between mice fed chicken protein ($n = 12$) and mice fed cod-scallop protein ($n = 10$)

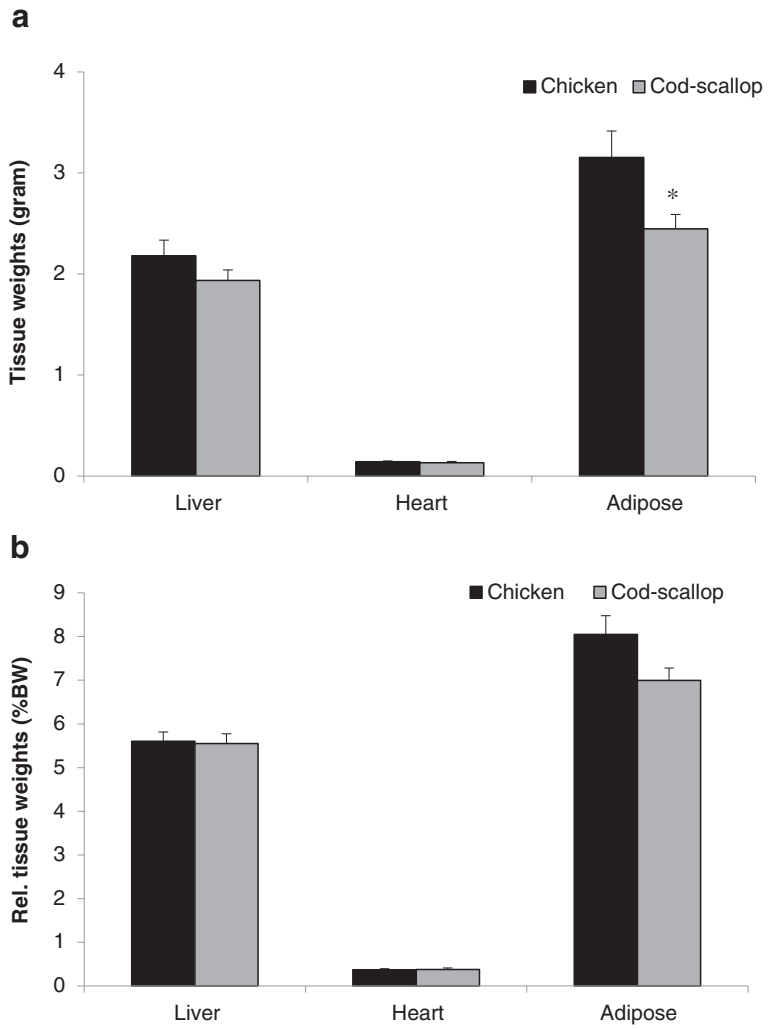

Fig. 2 Tissue weights and relative tissue weights of female apolipoprotein E-deficient mice. The mice were fed diets containing different protein sources for 13 weeks. Data are mean ± SEM. **a** Data from liver and visceral adipose tissue were analyzed using the independent samples *t*-Test. Data from heart were analyzed using the non-parametric Mann–Whitney *U* test. * denotes significant difference in tissue weight between mice fed chicken protein ($n = 12$) and mice fed cod-scallop protein ($n = 10$). **b** Data from liver were analyzed using the independent samples *t*-Test. Data from adipose tissue were log transformed before analysis using the independent samples *t*-Test. Data from heart were analyzed using the non-parametric Mann–Whitney *U* test. * denotes significant difference in relative tissue weight between mice fed chicken protein ($n = 12$) and mice fed cod-scallop protein ($n = 10$)

fatty acid amount (fatty acid concentration x liver mass), the chicken fed mice had elevated amounts of total hepatic fatty acids (Table 5). The fatty acid compositions evaluated in liver tissues (Table 6) showed that the group receiving the cod-scallop diet had higher ($p < 0.001$) percentage of saturated fatty acids (SFA) compared to the chicken group (30 % and 25 %, respectively). The percentage of linoleic acid was similar (approximately 5.0 %), EPA was only detected in the cod-scallop group, and both DPA and DHA were higher ($p < 0.001$) in the cod-scallop fed group compared to the chicken fed group (0.7 and 0.5 % DPA and 3.7, and 1.2 %DHA, respectively). However, when presented as mg/g liver tissue, the group receiving the cod-scallop diet had similar levels of SFA, lower levels of MUFA, and higher levels of EPA, DPA and DHA. The n6/n3 ratio was higher in the chicken fed group (4.3 vs 1.2) and the opposite was true for SFA/monounsaturated fatty acids (MUFA) ratio (0.4 in the chicken fed group and 0.6 in the cod-scallop fed group). The 16:1n7/16:0 ratios were 0.20 and 0.15 for chicken fed and cod-scallop fed mice, respectively.

Serum cholesterol and lipid levels

Serum LDL-cholesterol was significantly lower in the cod-scallop fed mice compared to the chicken fed mice, 12.5 vs 14.0 mmol/l ($P < 0.05$), however, neither serum

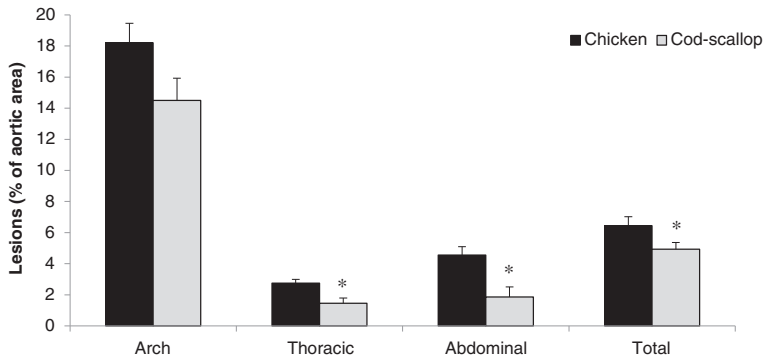

Fig. 3 Atherosclerotic plaque burden. The plaque burden is expressed as area percentage covered by lipid Oil Red O staining, in female apolipoprotein E-deficient mice fed diets containing different protein sources for 13 weeks. Data are mean ± SEM and were analyzed using the independent samples *t*-test. * denotes significant difference in atherosclerotic lesion between mice fed chicken protein ($n = 12$) and mice fed cod-scallop protein ($n = 10$)

concentrations of total cholesterol nor triglyceride differed significantly between the chicken and cod-scallop fed groups (Table 7).

Serum glucose, protein, cytokine, chemokine and hormone concentrations

Serum glucose concentrations differed between mice fed cod-scallop and chicken, being lower ($p < 0.05$) in the cod-scallop fed group (16.7mmol/l) compared to the chicken fed group (19.6mmol/l) (Table 7). Total protein content in serum did not differ between cod-scallop and chicken fed mice (95.4g/l and 86.8g/l, respectively) (Table 7). Leptin was higher ($p < 0.01$) in the chicken fed group (40.3pg/ml) compared to the cod-scallop fed group (25.4pg/ml) (Table 8). Serum levels of the cytokines and chemokines IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/GRO, TNF-α, MCP-1, and RANTES did not differ between the groups (data now shown). Three hours feed-deprived serum concentrations of insulin and glucagon did not differ between the two groups (Table 8). Insulin concentrations were 1.4ng/ml in both groups and glucagon concentrations were 700pg/ml and 1000pg/ml for chicken and cod-scallop fed mice, respectively.

Table 5 Lipid content and total fatty acids (FA) (mg/g) of hepatic tissue from apoE$^{-/-}$ mice

	Dietary treatments	
	Chicken ($n = 12$)	Cod-scallop ($n = 10$)
Lipid (mg/g liver)	430 ± 23	330 ± 35*
FA(mg/g fat)	194 ± 11	153 ± 16
FA (mg/g)*liver weight	423 ± 37	320 ± 28*

The mice were fed different protein sources for 13 weeks. Data are mean ± SEM ($n = 10$–12). Data were analyzed using the independent samples *t*-Test. *indicate significant difference ($p < 0.05$) between diet groups

Gene expression

The gene expression assays used for analysis of liver were chosen to include genes involved in cholesterol, lipoprotein and lipid metabolism, inflammatory response and endogenous antioxidant defense, possibly involved

Table 6 Fatty acid (FA) composition as percentage of total FA and mg/g of hepatic tissue from apoE$^{-/-}$ mice

	Dietary treatments			
	Chicken ($n = 12$)		Cod-scallop ($n = 10$)	
FA	%	mg/g	%	mg/g
14:00	0.98 ± 0.0	1.88 ± 0.1	0.87 ± 0.0	1.34 ± 0.2*
16:00	22.5 ± 0.3	43.6 ± 2.6	25.09 ± 0.6*	38.51 ± 4.0
16:1n7	4.61 ± 0.4	8.96 ± 0.9	3.74 ± 0.2	5.66 ± 0.5*
18:00	3.08 ± 0.1	5.91 ± 0.3	4.13 ± 0.2*	6.17 ± 0.5
18:1n9	45.03 ± 1.4	87.6 ± 6.0	41.95 ± 0.7*	64.44 ± 6.6*
18:1n7	5.12 ± 0.4	9.92 ± 0.9	3.55 ± 0.1*	5.38 ± 0.5*
18:2n6	4.93 ± 0.3	9.59 ± 0.8	4.96 ± 0.1	7.55 ± 0.7
20:1n9	0.63 ± 0.0	1.24 ± 0.1	0.42 ± 0.0*	0.60 ± 0.1*
20:4n6	1.25 ± 0.1	2.42 ± 0.2	1.09 ± 0.1	1.57 ± 0.1*
20:5n3	Bdl		0.95 ± 0.1	1.40 ± 0.1
22:5n3	0.52 ± 0.1	0.98 ± 0.1	0.71 ± 0.1*	1.04 ± 0.1*
22:6n3	1.18 ± 0.1	2.28 ± 0.2	3.66 ± 0.2*	5.38 ± 0.3*
SFA	24.63 ± 2.0	47.58 ± 4.7	30.09 ± 0.6*	46.02 ± 4.6
MUFA	55.40 ± 2.1	107.68 ± 7.7	49.54 ± 0.8*	75.92 ± 7.6*
n-3	1.70 ± 0.1	2.97 ± 0.3	5.31 ± 0.3*	7.82 ± 0.5*
n-6	6.18 ± 0.4	12.01 ± 1.0	6.05 ± 0.2	9.12 ± 0.8*
n6/n3	4.27 ± 0.3		1.2 ± 0.0*	
16:1n7/16:0	0.20		0.15	
Sum identified	87.78 ± 0.7	170.30 ± 10.3	91.04 ± 0.7	138.93 ± 4.7
Sum unidentified	12.22 ± 0.7	23.35 ± 1.4	8.97 ± 0.7	13.52 ± 2.1

The mice were fed diets containing different protein sources for 13 weeks. Data are mean ± SEM ($n = 10$–12). Data were analyzed using the non-parametric Mann–Whitney *U* test. *indicate significant difference ($p < 0.05$). Bdl, below detection limit of 0.5 area %

Table 7 Serum lipids, glucose and total protein concentrations in apoE$^{-/-}$ mice

	Dietary treatments	
	Chicken ($n = 12$)	Cod-scallop ($n = 10$)
Cholesterol (mmol/l)	24.20 ± 0.9	21.84 ± 0.7
LDL-Cholesterol (mmol/l)	13.97 ± 0.57	12.49 ± 0.37*
Triglycerides (mmol/l)	1.90 ± 0.2	2.16 ± 0.2
Glucose (mmol/l)	19.60 ± 1.0	16.68 ± 0.6*
Total protein (g/l)	95.40 ± 3.7	86.76 ± 4.5

The mice were fed diets containing different protein sources for 13 weeks. Data are mean ± SEM ($n = 10$–12). Data were analyzed with the independent samples *t*-Test. Data for triglycerides were analyzed using the non-parametric Mann–Whitney *U* test. *indicate significant difference ($p < 0.05$) between dietary treatments

in several phases of the atherosclerotic process (Table 9). Hepatic expression of the genes encoding the antioxidative protein paraoxonase 2 (*Pon2*) and vascular cell adhesion protein 1 (*Vcam1*) were down regulated in mice fed cod-scallop compared to mice fed chicken. Hepatic expression of all the other investigated genes were unaffected by the dietary intervention. The effects of the two diets on the cardiac function and homeostasis were also investigated by means of gene expression analysis of a battery of genes related to heart function, fibrosis and apoptosis. There were, however, no significant differences between cardiac gene expression of mice fed chicken or cod-scallop (Table 9).

Discussion

The main purpose of this study was to evaluate the effect of different protein sources on atherosclerotic development using female apoE$^{-/-}$ mice fed a Western-type diet. The protein sources were chicken or cod and scallop (1:1 on nitrogen basis). The reason for combining cod and scallop was to create a lean marine protein source high in taurine and glycine. Chicken was chosen because it is considered a healthy and lean protein source of terrestrial origin [26]. Mice fed the cod-scallop diet for 13 weeks had significantly reduced aorta atherosclerotic plaque burden compared to chicken fed mice.

Table 8 Serum concentrations of glucagon, insulin and leptin in apoE$^{-/-}$ mice

	Dietary treatments	
	Chicken ($n = 12$)	Cod-scallop ($n = 10$)
Glucagon (pg/ml)	698 ± 202	1054 ± 333
Insulin (ng/ml)	1.4 ± 0.4	1.4 ± 0.5
Leptin (pg/ml)	40.3 ± 11.6	25.4 ± 8.0*

The mice were fed diets containing different protein sources for 13 weeks. Data are mean ± SEM ($n = 10$–12). Data for leptin were analyzed using the independent samples *t*-Test. Data for insulin were log transformed before analysis using the independent samples *t*-Test. Data for glucagon were analyzed using the non-parametric Mann–Whitney *U* test. * indicate significant difference ($p < 0.05$) between dietary treatments

Table 9 Relative gene expressions in liver and heart of apoE$^{-/-}$ mice

Gene	Chicken	Cod-scallop	Gene	Chicken	Cod-scallop
Liver			Heart		
Cytokines and chemokines			*Cytokines and chemokines*		
Ccl2	*1.0 ± 0.11*	*0.85 ± 0.05*	Ccl2	*1.0 ± 0.12*	*0.80 ± 0.10*
Il1b	*1.0 ± 0.13*	*0.86 ± 0.05*	Ilb1	*1.0 ± 0.16*	*1.20 ± 0.38*
Icam1	*1.0 ± 0.13*	*0.97 ± 0.05*	Icam1	*1.0 ± 0.06*	*1.03 ± 0.11*
Vcam1	*1.0 ± 0.06*	*0.81 ± 0.06**	*Cardiac function and homeostasis*		
Endogenous antioxidant system			Agtr1	*1.0 ± 0.08*	*1.09 ± 0.13*
Pon2	*1.0 ± 0.04*	*0.84 ± 0.04**	Ankrd1	*1.0 ± 0.13*	*1.03 ± 0.24*
Nfe212	*1.0 ± 0.05*	*0.94 ± 0.04*	Nppa	*1.0 ± 0.31*	*1.17 ± 0.43*
Sod1	*1.0 ± 0.05*	*1.04 ± 0.07*	Nppb	*1.0 ± 0.21*	*1.07 ± 0.34*
Sod2	*1.0 ± 0.03*	*1.05 ± 0.03*	Myh6	*1.0 ± 0.04*	*1.10 ± 0.08*
Gpx1	*1.0 ± 0.05*	*1.01 ± 0.04*	Myh7	*1.0 ± 0.15*	*0.80 ± 0.12*
Gpx4	*1.0 ± 0.04*	*1.04 ± 0.04*	*Angiogenesis*		
Ucp2	*1.0 ± 0.13*	*0.94 ± 0.08*	Vegfb	*1.0 ± 0.06*	*1.07 ± 0.15*
Cholesterol and lipoprotein metabolism			*Apoptosis*		
Abcg5	*1.0 ± 0.26*	*0.89 ± 0.09*	Bcl2	*1.0 ± 0.07*	*0.99 ± 0.07*
Abcg8	*1.0 ± 0.25*	*0.77 ± 0.07*	Casp3	*1.0 ± 0.08*	*1.14 ± 0.14*
Acat2	*1.0 ± 0.21*	*0.95 ± 0.05*	*Fibrosis*		
ApoB	*1.0 ± 0.18*	*0.98 ± 0.05*	Col1A1	*1.0 ± 0.06*	*1.10 ± 0.12*
Cyp7a1	*1.0 ± 0.26*	*0.89 ± 0.06*	Col3A1	*1.0 ± 0.06*	*1.01 ± 0.09*
Hmgcr	*1.0 ± 0.08*	*1.05 ± 0.07*	Fn1	*1.0 ± 0.04*	*1.07 ± 0.14*
Ldlr	*1.0 ± 0.25*	*0.81 ± 0.05*	Timp1	*1.0 ± 0.24*	*0.89 ± 0.15*
Srb1	*1.0 ± 0.52*	*0.37 ± 0.02*			
Vldr1	*1.0 ± 0.20*	*1.05 ± 0.09*			
Nitrogen and fatty acid metabolism					
Cps1	*1.0 ± 0.09*	*1.17 ± 0.08*			
Cpt1a	*1.0 ± 0.06*	*0.98 ± 0.06*			
Acox1	*1.0 ± 0.05*	*1.05 ± 0.02*			
Acaca	*1.0 ± 0.17*	*1.01 ± 0.19*			
Scd1	*1.0 ± 0.31*	*1.91 ± 0.30*			
Adipoq	*1.0 ± 0.58*	*0.99 ± 0.07*			
Adipor	*1.0 ± 0.05*	*0.93 ± 0.02*			
Ffar1	*1.0 ± 0.11*	*1.11 ± 0.06*			

The mice were fed diets containing different protein sources for 13 weeks. Data are mean ± SEM ($n = 10$–12)
*Indicate significant difference ($p<0.05$) between dietary treatments.

Diets rich in seafood are generally recommended to humans due to the triglyceride lowering and anti-inflammatory effects of EPA and DHA [27]. However, in the present study, the serum concentration of triglycerides did not differ significantly between the two groups. A triglyceride lowering effect of EPA and DHA has mainly been shown at pharmaceutical doses and is not commonly evident at nutritional levels [28] as given in the present study. When the cholesterol content in the

two diets was analyzed, it was found that the chicken diet contained 0.20 % cholesterol whereas the cod-scallop diet contained 0.19 % cholesterol, but this small difference is not sufficient to cause a reduction in aorta atherosclerosis *per ce*. Taurine is known to reduce cholesterol levels in blood by increased cholesterol clearance from blood circulation, bioconversion of cholesterol to bile acid in liver and excretion of cholesterol and bile acids from the intestine [29]. Hence, the higher dietary level of taurine in the cod-scallop diet was expected to contribute to a reduced level of circulating cholesterol. Indeed, a reduced level of serum LDL cholesterol was observed in the cod-scallop fed mice compared to the chicken fed mice, whereas we did not find a significant reduction of serum total cholesterol in the cod-scallop fed mice compared to the chicken fed mice.

The mice fed chicken protein gained more weight than mice fed cod-scallop protein and body weight, visceral white adipose tissue weight and liver lipid content was higher in chicken fed mice compared to cod-scallop fed mice. A positive correlation between satiety and blood taurine concentrations in humans has been reported [30], and it has been demonstrated that taurine supplementation of male mice affected energy intake and prevented a high-fat diet induced obesity [31]. In this study, however, the feed consumption was equal in the two groups suggesting that metabolism was affected differently. In a recent study by Tastesen et al. [17], it was shown that dietary taurine and glycine correlated inversely with body fat mass. The mechanisms, however, remain unknown. Choice of dietary protein source has been documented to affect energy metabolism and to be associated with diet-induced obesity in rats [32, 33]. Rats fed fish protein hydrolysates have been shown to have elevated plasma bile acids and reduced visceral adipose tissue relative to a casein fed control group [32, 33]. The plasma concentrations of bile acids were not measured in this study, hence the exact mechanisms behind the weight gain difference, were not elucidated.

Furthermore, the cod-scallop fed mice had lower concentration of leptin compared to chicken fed mice. This is most probably due to the lower content of adipose tissue, which is the primary site of leptin production. Leptin is a key hormone in regulation of food intake and energy expenditure [34]. Lately it has also been demonstrated to play a direct role in almost every step in the development of atherosclerotic plaques [35]. It has previously been reported for both humans and rodents that obesity is associated with leptin resistance and that obese individuals have elevated leptin levels without any down-regulation of food intake [36]. Leptin is also indicated to function as a proinflammatory adipokine, and to further investigate the effect on the inflammatory response, we analyzed both circulating levels and hepatic expression of several inflammatory markers. Expression of the *Vcam1* gene encoding the cellular adhesion molecule VCAM-1 was lower in the cod-scallop fed mice. This protein mediates firm adhesion of leukocytes that then migrate into the sub-endothelium. The *Vcam1* gene is normally up regulated by hyperlipidemia and by inflammatory cytokines [37], proposing a potentially higher inflammatory condition in the chicken fed mice. However, hepatic expressions of inflammatory marker genes such as *Ccl2*, *Il1b*, and *Icam1* were not influenced and serum levels of inflammatory proteins were unaltered between the two groups. The level of atherosclerotic plaque formation observed in this study represents levels found in asymptomatic, apparently healthy humans [38] and not surprisingly the serum levels of cytokines and chemokines are unaffected by the dietary intervention in this model.

It is well accepted that oxidative stress may accelerate the development of atherosclerosis [39] with subsequent up-regulation of cellular antioxidants such as the protein PON2 [40]. In the cod-scallop fed mice the expression of the *Pon2* gene was lower compared to chicken fed mice, suggesting lower oxidative stress, which may be a possible explanation for the reduced atherogenesis. Taurine is known to possess antioxidative capacity [41] and may have attributed to the lower oxidative stress in the cod-scallop fed group.

In the present study, the dietary concentrations of n-3 PUFAs were higher, and of SFA and MUFA were lower in the cod-scallop diet as compared to in the chicken diet. Compared to the chicken fed mice, the mice fed cod-scallop diet had similar liver levels of SFA, lower levels of MUFA and higher levels of EPA, DPA and DHA, and further a correspondingly lower n-6/n-3 ratio. An antiatherosclerotic effect of marine n-3 PUFA have previously been observed both in the apoE$^{-/-}$ mouse model [21] and in the Ldlr$^{-/-}$ mouse model [42–45]. As previously mentioned, the amounts of n-3 PUFA given in the present study were lower compared to the previous studies. However, we cannot exclude the possibility that the dietary fatty acid composition contributed to the observed effects in the present study.

In a clinical human setting, atherosclerosis leads to hemodynamic stress often followed by a compensatory hypertrophic growth of the heart and ultimately plaque rupture and ischemic heart disease. To search for detectable differences in cardiac gene expression of markers of heart function and homeostasis, fibrosis, apoptosis and angiogenesis, RNA was isolated from cardiac apices from the mice. No differences were found between mice fed chicken and mice fed cod-scallop in any of the tested genes, indicating that the diets did not affect the heart. This was supported by the lack of hypertrophic growth of the cardiac muscle. There is however little evidence

indicating that the heart would be affected considering the low levels of dietary cholesterol given to the mice and the low atherosclerotic burden observed in the mice, and substantial effects on the heart was not expected.

Conclusions

This study has demonstrated a beneficial metabolic effect in the mice fed cod-scallop compared to the mice fed chicken, as aorta atherosclerotic burden, body weight, visceral adipose tissue, serum glucose and leptin levels, all were reduced. The results warrant further investigations to elucidate in detail the molecular mechanisms underlying the observed effects.

Additional file

Additional file 1: Table S1. Predesigned TaqMan® Gene Expression assays used. (DOCX 16 kb)

Competing interests

The authors declare no competing interest, neither financial nor non-financial.

Authors' contributions

Karl-Erik Eilertsen (KEE), Bjørn Liaset (BL) and Edel O. Elvevoll (EOE) conceived and designed the study, Ida-Johanne Jensen (IJJ), KEE and Mari Walquist (MW) performed the experiments, IJJ and KEE analyzed the data. IJJ and KEE wrote the paper. All authors contributed to the finalization of the manuscript.

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