UiT
THE ARCTIC
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Faculty of Health Sciences

Cardiovascular Research Group

Calanus oil and its lipid constituents

Impact on obesity and obesity-related metabolic disorders in rodents

Anje Christina Höper

A dissertation for the degree of Philosophiae Doctor – December 2013

UNIVERSITY OF TROMSØ

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Contents

ACKNOWLEDGEMENTS

This thesis was carried out at the Cardiovascular Research Group, University of Tromsø, with the main financial support from the University of Tromsø. My six-months' stay at the Quebec Heart and Lung Institute was supported by a scholarship from the University of Tromsø.

First of all I would like to express my deepest gratitude to my supervisor Terje S Larsen and my co-supervisor Ellen Aasum. Thank you for your scientific guidance, your unbeatable optimism, your patience and especially for always having an open door, no matter how many other things there were to do.

A big "thank you" to the entire Cardiovascular Research Group- "Family" without whom I never would have gotten through these years. The lunchroom has been the place of many get-togethers, reaching from purely academic to extremely social and "koselig", something I appreciated a lot.

Thanks to all of you who helped me in the lab, be it theoretically or practically as well as all co-authors of my papers. I will go with the Norwegian phrase "Ingen nevnt, ingen glemt", but you know who you are and what you have done for me!

A special thanks to Knut Steinnes for immediate response to computer-related and other technical difficulties, as well as Karin Akselsen for solving basically any paperwork-problem, always having a smile on her face and for the occasional chat about sewing patterns, fabric and other important things in life. A big thank you also to the entire staff of the "Department of Comparative Medicine" for taking care of my many four-legged "babies", helping with sample taking etc.

Thanks to Calanus A/S, for partial financial support of some of the studies. A special "Calanus-thanks" to Alice Pedersen for "biochemical support", a contagious smile and for sharing many research-unrelated creative ideas. Thank you also to Karl-Erik Eilertsen for shedding some light in the intricate world of long-chain fatty acids.

A special thanks to my office-"roomies" through all these years for lots of fun, moral support and (too?) many hours of both scientific and private discussions: Belal Aljabri, Neoma Boardman, Flavia Paunas, David Johansen, Wahida Salma, Selene Sollie, Stian Ludvigsen and Eirik Engstad.

For my little "semester abroad" (apart from thanking Terje and Ellen for letting me go), I would like to send a big overseas-hug to everybody in "Team Labo Marette" for making my stay unforgettable. Especially I would like to thank Bruno Marcotte for introducing me to the world of cell culture and being a great tutor for both lab-math and Quebec-French.

Last but not least, I would like to thank my family: my parents and brother for always believing in their "little one" and especially my dear Judeson for "playing the house-wife", not only in Canada, but also at home when times were rough. I couldn´t have done it without your support! The biggest thanks of all goes to our two sweethearts Linus and Charlotte, for being the wonderful daily reminders of what really matters in life!

LIST OF PAPERS

Paper I Höper AC, Salma W, Khalid AM, Hafstad AD, Sollie SJ, Raa J, Larsen TS, Aasum E. Oil from the marine zooplankton *Calanus finmarchicus* improves the cardiometabolic phenotype of diet-induced obese mice. *Br J Nutr 2013, 110 (12): 2186-2193*

Paper II Höper AC, Salma W, Sollie SJ, Hafstad AD, Lund J, Khalid AM, Raa J, Aasum E, Larsen TS. Wax esters from the marine copepod *Calanus finmarchicus* reduce diet-induced obesity and obesity-related metabolic disorders in mice. *J Nutr 2013 in press*

Paper III Höper AC, Larsen TS, Fuglesteg BN, Khalid AM, Tande KS, Aasum E, Pedersen AM, Olsen RL. Chemical composition of Calanus oil and safety assessment based on physiological studies in rats *(manuscript)*

ABBREVIATIONS

AA	arachidonic acid
ALA	α-linolenic acid
CLS	crown-like structures
DAG	diacylglycerol
DHA	docosahexaenoic acid
DIO	diet-induced obesity
ER	endoplasmatic reticulum
EPA	eicosapentaenoic acid
eWAT	epididymal fat (white adipose tissue)
FA	fatty acid
FAOH	fatty alcohol
GLP-1	glucagon-like peptide-1
GPR	G-protein coupled receptor
HFD	high-fat diet
IPGTT	intraperitoneal glucose tolerance test
IL	interleukin
LPS	lipopolysaccharide
MCP-1	monocyte-chemoattractant protein-1
MUFA	monounsaturated fatty acid
NEFA	non-esterified fatty acids
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
OGTT	oral glucose tolerance test
PUFA	polyunsaturated fatty acid
pWAT	perirenal fat (white adipose tissue)
SDA	stearidonic acid
SFA	Saturated fatty acid
TAG	triacylglycerol
TNFα	tumor necrosis factor α

SUMMARY OF THE THESIS

The prevalence of obesity has risen dramatically worldwide and has reached epidemic proportions. Particularly, visceral or abdominal obesity has been shown to correlate strongly with a number of pathologies, such as type 2 diabetes mellitus, cardiovascular disease and stroke. Consumption of fish and marine oils containing omega-3 polyunsaturated fatty acids (n-3 PUFAs) has been shown to be beneficial in some of these conditions. Oil from the marine zooplankton *Calanus finmarchicus* (Calanus oil) has recently emerged as a nutritional supplement for the human market, and the aim of this thesis was to provide data on the chemistry, safety issues, as well as on the physiological effects of this oil in obese rodents. Unlike other n-3 PUFA-rich nutritional supplements, Calanus oil is mainly composed of monoesters of long-chain fatty acids and fatty alcohols, also called wax esters. In addition, it is rich in the potent anti-oxidant astaxanthin. The fatty acid moiety of the wax esters includes high amounts of n-3 PUFAs (EPA, 20:5; DHA, 22:6, stearidonic acid=SDA, 18:4), as well as monounsaturated fatty acids (MUFAs), such as gondoic (20:1 n-9) and cetoleic acid (22:1 n-11). Toxicology analysis confirms low levels of heavy metals, organic and non-organic impurities. Calanus oil supplementation (1.5%, w/w) had no adverse effects during high-fat feeding in rats or mice. On the contrary, it provided beneficial health effects compared to animals fed a high-fat diet alone, as demonstrated by (1) reduced body weight gain, (2) reduced deposition of intra-abdominal fat, (3) reduced adipose tissue inflammation, (4) reduced hepatic steatosis and (5) improved glucose tolerance. Almost identical effects were seen after supplementation with Calanus oil-derived wax esters (1%, w/w). Wax ester supplementation also improved aerobic capacity compared to high-fat diet alone. By comparison, supplementation with purified EPA+DHA ethyl esters in a concentration corresponding to the total amount of n-3 PUFAs in the wax ester diet showed primarily anti-inflammatory effects, whereas the impact on obesity and glucose tolerance was only modest. In summary, Calanus oil as a dietary supplement is well tolerated and exerts beneficial effects against obesity and obesity-related disorders in rodents. Although the biologically active components are not identified, they seem to be confined to the main lipid fraction (wax esters) of the oil. Most likely, its beneficial effects are dependent on an interaction between different MUFAs and PUFAs, as well as on a rather slow digestion and uptake into the circulation at the distal part of the intestine. Calanus oil seems to be a good alternative to other n-3 PUFA containing supplements, but has the advantage of being naturally low in environmental contaminants and effective in very small amounts.

1. INTRODUCTION

1.1 Obesity and obesity-related disease

The prevalence of obesity has increased dramatically world-wide during the past 2-3 decades, not only in industrialized[1], but also in developing countries adopting Western life-style and food habits [2]. There are no indications that the present obesity pandemic is under control or indications that it will change to the better.

Obesity has a number of consequences for human health, such as insulin resistance, type 2 diabetes, cardiovascular disease, stroke, and early, sudden death [3]. Clustering of a number of cardiovascular risk factors in obese humans is often seen and has resulted in different terms such as "metabolic syndrome" [4, 5] "multiple risk factor clustering syndrome" [6] or simply called "cardiometabolic risk", including the components insulin resistance, hypertension, hypertriglyceridemia, reduced HDL-cholesterol and increased abdominal obesity. Many of the risk factors occurring in these conditions can be reduced by weight loss. Excess fat around intra-abdominal organs, referred to as visceral or (intra-) abdominal obesity, represents a greater risk factor for mortality and morbidity in humans than general obesity [7], and abdominally obese individuals are particularly prone to developing diabetes [1]. Japan has addressed the importance of visceral adiposity to the metabolic syndrome by prioritizing lifestyle interventions to reduce visceral adiposity over drug treatment in their national guidelines [8].

The increased cardiometabolic risk of excess visceral fat is most probably due to differences in physiological, biochemical and molecular properties of visceral and subcutaneous adipose tissue. For example, compared to adipocytes from subcutaneous fat depots, visceral adipocytes have been shown to have a higher lipolytic response to catecholamines [9], to be less responsive to insulin [10, 11] and having reduced expression of the anti-diabetic and anti-inflammatory hormone adiponectin [12] and its receptors [13].

1.2 Inflammation and insulin resistance

Abdominal obesity is strongly associated with insulin resistance, a condition that, if not halted, ultimately leads to fully developed type 2 diabetes. The mechanisms of insulin resistance are multifactorial and still under investigation, but it seems clear that low-grade

inflammation, especially locally in abdominal adipose tissue, together with a constant nutrient excess play the key role.

The usual series of events in the case of inflammation are initiation (by infection, trauma etc.) followed by an immune response (infiltration of neutrophils, macrophages and other components of the immune system), and ultimately resolution of inflammation (Figure 1). While previously considered a passive event, it has now become evident that active processes steer the resolution process. This has led to intensified research in the field of pro-resolving agents [14]. If the triggering event is not removed, such as nutrient excess in adiposity, and pro-resolving factors are not sufficient to terminate the inflammation, a persistent (low-grade) inflammation develops (Figure 1). To emphasize the special etiology of inflammation in obesity, the term "metaflammation" has been coined by Hotamisligil and colleagues [15].

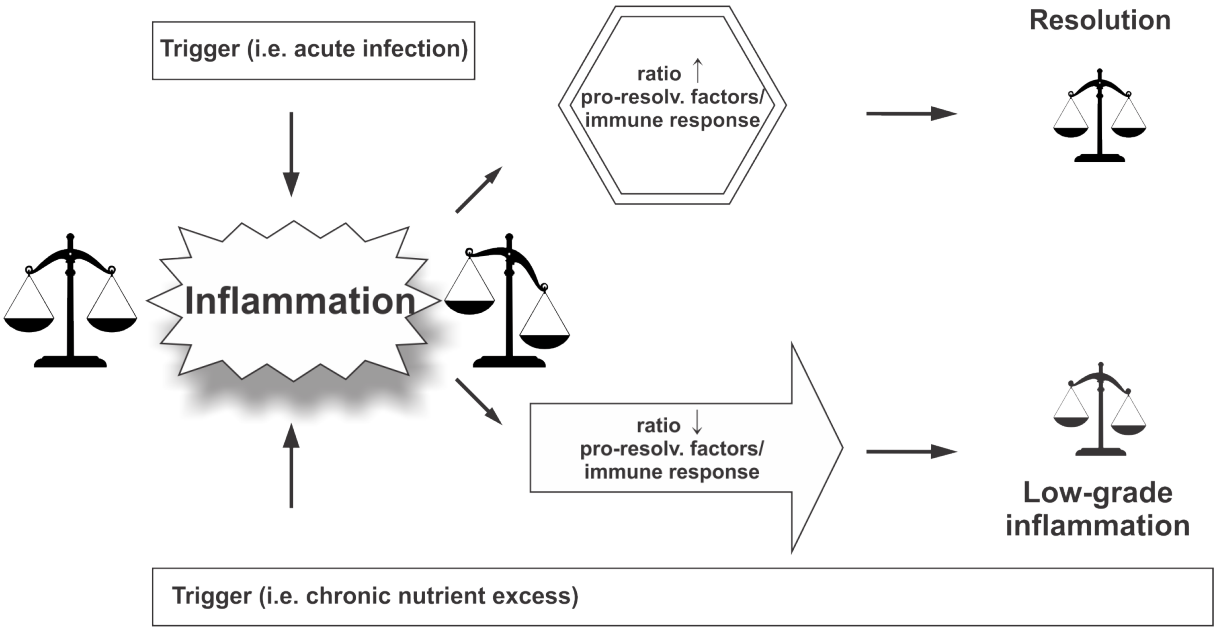

Figure 1. Course of acute vs. chronic inflammation. A trigger causes inflammation with the recruitment of pro-inflammatory cells and mediators, summarized as "immune response". Pro-resolving factors antagonize the immune response actively and, if their action is sufficient, it results in complete resolution (upper pathway). If not, the immune response is blunted but not removed, and a low-grade inflammation persists (lower pathway).

Obesity leads to enlarged abdominal adipose tissue, in which a local inflammatory response is initiated, including increased production of pro-inflammatory cytokines (i.e. TNF-α, IL-6 and IL-1β) and chemokines, such as monocyte chemoattractant protein-1 (MCP-1).

There is an ongoing debate regarding the triggering event for inflammation in adipose tissue, but the literature points to hypoxia and nutrient excess as the two main factors[15-17].

Reduced tissue pO_2 has been measured in adipose tissue of both obese animals [18] and humans [19] and different explanations exist. As adipose tissue expands, it does so primarily by hypertrophy, i.e. an increase in size of the individual adipocytes. Hypertrophic cells lead to insufficient oxygen diffusion, due to increased distances between the blood-bearing vessels [20], which in turn results in local hypoxia. Other possible causal factors of adipose tissue hypoxia include reduced blood flow, reduction of capillary density and/or increased vasoconstriction in obese vs. non-obese adipose tissue [17]

The release of pro-inflammatory markers and chemokines increases the amount of macrophages (and other inflammatory cells) in the adipose tissue, mainly by recruitment of new macrophages by MCP-1. Macrophages that are usually present in the adipose tissue switch from an anti-inflammatory (M2) state to a pro-inflammatory (M1) state, thereby potentiating the inflammatory process [21]. Dead adipocytes get surrounded by M1-type-macrophages and form so-called "crown-like structures" (CLS), a typical picture in immune-histological sections from obese adipose tissue [22].

Nutrient excess and quality can modulate inflammation and insulin sensitivity directly or indirectly (Figure 2); Nutrient metabolites, such as lipid intermediates, can provoke pro-inflammatory pathways, e.g. via toll-like receptors [15], but also directly impair insulin signaling [16, 23]. Inflammation, in turn can increase lipid intermediates by increasing lipolysis, thereby potentiating the negative influence on insulin signaling.

Recently, obesity and nutrient quality have also been linked to certain gut microbiota phenotypes [24]. Interestingly, obese/high fat-fed animals have a constantly higher intestinal permeability, while at the same time showing higher serum levels of the bacterial endotoxin lipopolysaccharide (LPS), possibly related to a change towards increased LPS-containing microbiota [25, 26].

Hypoxia and nutrient excess are also suspected to be triggers of endoplasmatic reticulum (ER) stress, also called “unfolded protein response”- UPR. The ER is not only involved in protein synthesis and folding, but also functions as a lipid and nutrient sensor. ER stress is an adaptive mechanism, but can ultimately lead to cell death if the underlying trigger is not removed or is too excessive. Vicious circles can occur where inflammation and insulin

resistance activate ER stress, which in turn increases inflammation and thus further impairs insulin signaling [16, 27, 28].

A local inflammatory response has also been shown in the liver, contributing to increased gluconeogenesis and thereby to worsening of hyperglycemia. In addition, inflammatory mediators such as cytokines and acute phase proteins produced by the liver are contributing factors to systemic inflammation. Taken together, whatever the trigger and wherever the origin, the role of inflammation as a link between obesity and insulin action has become evident [15, 28, 29].

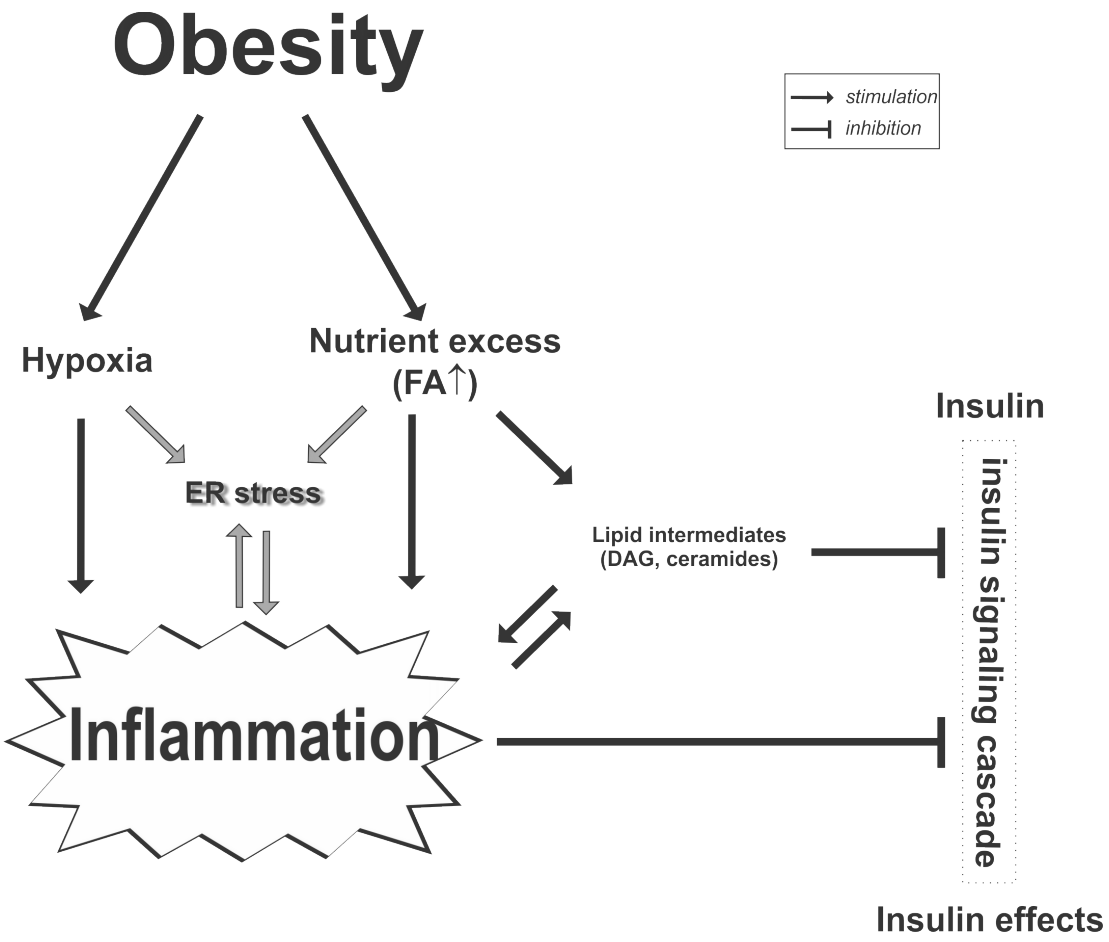

Figure 2. Relationship between obesity, inflammation and insulin resistance. In adipose tissue, obesity leads to constant nutrient excess as well as local hypoxia. Both factors can induce inflammation, either directly or via ER stress. Lipid intermediates such as diacylglycerol (DAG) or ceramides, resulting from increased fatty acid (FA) levels, can increase inflammation. On the other hand, inflammation can increase lipid intermediates by increased lipolysis. Inflammation and lipid intermediates can directly interfere with insulin signaling and finally lead to insulin resistance.

1.3 Measures against the obesity epidemic

Lifestyle interventions, such as increased exercise and calorie-reduced diets, are undoubtedly effective in reducing obesity. Unfortunately, the long-term success rate of these interventions is very poor. Interestingly, change of food patterns, e.g. increased intake of less refined carbohydrates, are shown to have beneficial effects on cardiometabolic risk factors, independent of weight loss[30]. This has contributed to a change of focus from pure quantity to quality, and to the development of the "neutraceutical" and "functional food"-industry [31, 32]. One of the emerging research fields over the past years is that of marine products with potential health benefits.

1.4 Nutrition and fatty acid classes

It has long been known that a high intake of saturated as well as trans-fatty acids is harmful, especially for cardiovascular health, and their replacement by mono- and polyunsaturated fatty acids (MUFAs and PUFAs, respectively) has been implicated in recommendations from institutions like the American Heart Association[33]. However, it has become evident that a generalization of PUFAs is inadequate due to the different biological mechanism of its subclasses as discussed in the following.

1.4.1 Polyunsaturated fatty acids (PUFAs)

The two main classes of PUFAs are the omega-3 and omega-6 polyunsaturated fatty acids (n-3 PUFAs and n-6 PUFAs, respectively). The ratio of n-6 to n-3 PUFAs in our diet has risen from close to 1:1 in the times of hunters and food-gatherers to today's values of 15-20:1 [34]. There is considerable evidence showing that a high content of n-6 PUFAs in the diet, relative to n-3 PUFAs, is a predisposing factor for obesity [35], and very recently an updated meta-analysis could show that, contrary to prior belief, n-6 PUFAs have no beneficial cardiovascular effects [36].

Replacing n-6 by n-3 PUFAs or simply increasing the intake of n-3 PUFAs in the diet has proven to be beneficial, resulting in recommendations of a n-6/n-3 ratio of 4:1 to5:1[37]. Most research on n-3 PUFAs has been done on eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) which are the "typical marine" n-3 PUFAs. But also

the plant-derived alpha-linolenic acid (ALA; 18:3 n-3), which is the common precursor for EPA and DHA, is a widely investigated n-3 PUFA. n-3 and n-6 PUFAs share the same enzymes involved in the metabolic pathway for conversion of these fatty acids (see Figure 3). Therefore, excess of one fatty acid family can interfere with the metabolism of the other, significantly reducing its conversion and thereby the biologic action of the metabolite(s). n-3 PUFAs have potent anti-inflammatory effects. Accordingly, n-3 PUFA supplementation has been proven to be beneficial in different pathologies like cardiovascular disease, rheumatoid arthritis, diabetes mellitus and neurological diseases, many of which are related to inflammation. One of the suggested underlying mechanisms for the anti-inflammatory effects is the reduction of the n-6 PUFA arachidonic acid (AA; 20:4 n-6) via substrate competition. Since AA is correlated with a high pro-inflammatory activity, due to its potential to generate pro-inflammatory eicosanoids (prostaglandins, leukotrienes, thromboxanes etc; Figure 3) [38, 39], its reduction implies decreased inflammation.
Other suggested mechanisms behind n-3 PUFA-mediated reduction in inflammatory activity include inhibition of the pro-inflammatory NFkB signalling pathway, increased production of pro-resolving mediators (such as resolvins, protectins or maresins) [40] and activation of the novel G-protein coupled receptor (GPR) 120 [41, 42].

Resolvins, protectins and maresins are newly emerged families of mediators derived from EPA and DHA, which are potent in very low concentrations (pg-ng) [43]. GPR 120 is a recently identified receptor for n-3 PUFAs. It is highly expressed on adipocytes [41, 44] and pro-inflammatory macrophages [41], but also in the intestine [41, 45], especially in entero-endocrine cells [45, 46]. GPR 120 has been shown to exert anti-diabetic and anti-inflammatory actions [41]. Taken together, different mechanisms of action, both via anti-inflammatory mechanisms (i.e. reduction of AA-derivates and GPR 120-activation) as well as via the pro-resolving axis can potentiate beneficial effects of n-3 PUFA on inflammation.

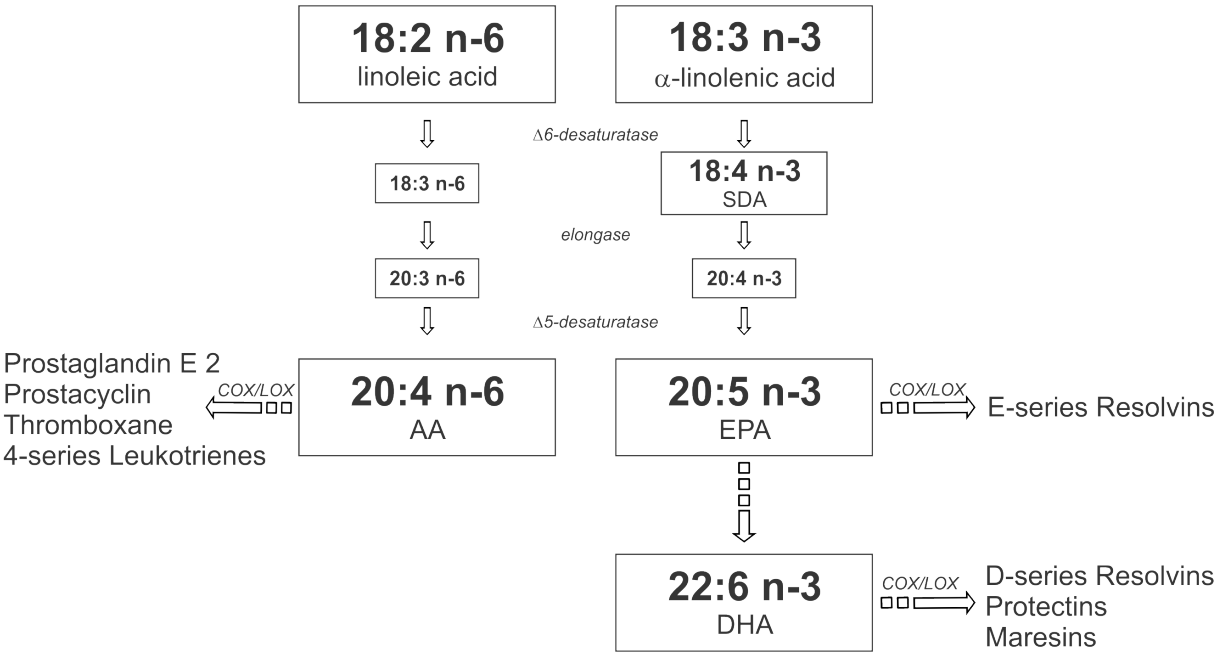

Figure 3. Simplified metabolic pathway for conversion of n-6 and n-3 polyunsaturated fatty acids (PUFAs), rendering predominantly pro-inflammatory mediators derived from n-6 PUFAs (left hand side) and pro-resolving (thereby indirectly anti-inflammatory) mediators derived from n-3 PUFAs (right hand side). Cyclooxigenases (COX) and lipoxigenases (LOX) catalyze the production of these mediators. Resolvins are named after the initial letter of their precursor n-3 PUFA: E-series for eicosapentaenoic acid (EPA)-derived, D-series for docosahexaenoic acid (DHA)-derived resolvins. AA=arachidonic acid, SDA=stearidonic acid

Even though the human body can synthesize EPA and DHA from its precursor ALA, the conversion is extremely inefficient due to the rate-limiting enzyme Δ^6desaturase (Figure 3)[47]. Therefore, direct supplementation with EPA and/or DHA is the preferential mode of increasing incorporation of these beneficial PUFAs in human tissue, but intake in the general population is often far below recommended levels [48]. Stearidonic acid (SDA; 18:4 n-3), the first metabolite formed directly from ALA by the rate-limiting enzyme Δ^6desaturase, is an alternative for oral n-3 PUFA substitution[48]. It is rapidly and more efficiently than ALA converted into longer n-3 PUFAs [49-51]and has been shown to successfully increase the so-called "omega-3 index" [52]. This index is a surrogate measure for the incorporation of EPA and DHA in tissues and can be considered as a cardiovascular risk marker, as tissue incorporation is considered to be the primary step of a variety of cardioprotective mechanisms [53].

1.4.2 Monounsaturated fatty acids (MUFAs)

Since the "Seven Countries study" first pointed out the diet from the Mediterranean region as beneficial for the reduction of cardiovascular risk [54], a lot of research has been done on finding the active compound(s). The Mediterranean diet is rich in olive oil, which has a high

percentage of the MUFA oleic acid (18:1 n-9). Therefore, researchers have proposed an important role of MUFA for cardiovascular health. In fact, MUFAs are reported to have favorable effects on metabolic syndrome and markers of cardiovascular disease risk such as blood pressure, blood lipids and insulin sensitivity, as well as the potential to ameliorate obesity risk [55]. The focus of MUFA research has mainly been on oleic acid, whereas others, i.e. gondoic (20:1 n-9) or cetoleic acid (22:1 n-11), have hardly been investigated, despite their relative abundance in some fish types. However, these two long-chain marine-derived MUFAs are of great interest, as recent studies by a Japanese group have shown beneficial effects on obesity and obesity-related disorders in mice [56-58].

1.5 Calanus oil and wax esters

The marine zooplankton *Calanus finmarchicus*, a member of the copepod-family, is the most abundant crustacean and one of the dominating food sources for fish in the North Atlantic [59]. The annual biomass production is enormous, and, as only a minor fraction of it is being utilized by fish and sea mammals [60], vast amounts are available for direct harvesting, representing a novel raw material for the Norwegian biomarine industry. *Calanus finmarchicus* is feeding on phytoplankton and stores energy mainly as oil, which makes up to 50 % of its dry weight [61]. As *Calanus finmarchicus* is situated at a low trophic level of the marine ecosystem, Calanus oil contains very low, if any, levels of environmental pollutants often found in organisms from higher trophic levels [62].

Calanus oil is rich in the same n-3 PUFAs as those found in other marine oils - EPA and DHA – but contains in addition high amounts of SDA as well as other long-chain FA such as the MUFA gondoic acid (20:1 n-9) and cetoleic acid (22:1 n-11) [63, 64] .
With only a few exceptions, those fatty acids (FAs) are not found, or found in small quantities in other marine oils. The exact percentages of FA-contents are difficult to predict as they fluctuate with time point and location of harvesting [61, 65]. Table 1 shows the composition of Calanus oil (average of >3 batches of oil harvested in different years).

	g/100 g oil
SFA	14.7
MUFA	14.0
PUFA	
n-3 FA	19.2
n-6 FA	1.2
FAOH	39
Sterols	0.5
Astaxanthin	0.1
Others	11.3

Table 1. Concentrations of the main components of Calanus oil**.** Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), fatty alcohols (FAOH)

While the FAs in fish and marine mammals are mostly bound as triacylglycerol, they are found primarily in phospholipids in Antarctic krill (*Euphasia superba*). In *Calanus finmarchicus,* on the other hand, the majority of the FAs (about 80%) is esterified to unsaturated fatty alcohols as so-called wax-esters.

Wax esters in general are FAs esterified to fatty alcohols (FAOHs). In copepods such as *Calanus finmarchicus*, wax esters represent a long-term energy storage and regulate buoyancy [66]. In Calanus oil, the FAs have a carbon chain length ranging from 14-22, including both saturated fatty acids (SFAs), MUFAs and PUFAs. The FAOHs are mostly monounsaturated with a carbon chain length of 16-22 [67], comprising wax esters with a length between 30-44 carbon atoms.

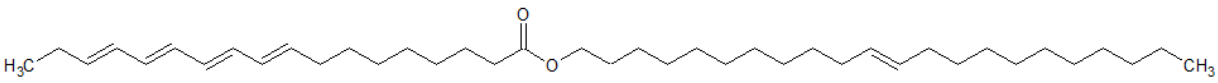

Figure 4. A typical wax ester in Calanus oil with the polyunsaturated omega-3 fatty acid SDA (18:4 n-3) and a long-chain monounsaturated alcohol (22:1 n-11)

Apart from its high amount of wax ester-bound unsaturated fatty acids, Calanus oil contains a number of other components, including phytosterols and anti-oxidants such as astaxanthin. Phytosterols have been approved by both American and European Food safety authorities as cholesterol-lowering food additives in margarines [68, 69]

The carotenoid astaxanthin is one of the strongest anti-oxidants found in nature [70], providing protection of stored lipids both in *Calanus finmarchicus* and other copepods [71]. Astaxanthin has been attributed potential health benefits in a number of diseases, such as cancer, chronic inflammatory and neurodegenerative conditions as well as cardiovascular- and metabolic diseases [72].

2. AIM OF THE STUDY

Despite its high content of potentially health-promoting compounds and its enourmous biomass in the North Atlantic, Calanus finmarchicus has not been investigated for its possible health effects until recently; Eilertsen et al.[73] showed that Calanus oil supplementation decreased plaque formation in the aorta of apoE-deficient mice. Beyond that, no health-promoting effects of Calanus oil have been reported in the literature.

Given the rising prevalence of obesity and obesity-related disorders, the aim of this study was to investigate the effect of Calanus oil on diet-induced obesity and its metabolic disorders. As the wax ester-binding of fatty acids is a unique feature that distinguishes Calanus oil from other marine oils, the study was also designed to examine the role of Calanus oil-derived wax esters. Furthermore, in the light of a potential use as a health supplement for humans, data on toxicology and safety of Calanus oil were obtained.

3. METHODOLOGICAL CONSIDERATIONS

3.1 Animal studies

In this study we used Wistar rats and C57Bl/6 mice. C57Bl/6 mice are the best characterized inbred mouse strain, and it is widely used in high fat-feeding studies, as these animals are highly prone to diet-induced obesity (DIO) and diabetes. In line with many other groups (and commercially available DIO mice) we used the so-called "van Heek-series" diets [74]. The lard-based high-fat diet (HFD) contained 45% energy from fat (Test diet 58V8, corresponding to the original D12451 from Research Diets) and was used for both our diet-induced overweight controls (HFD) and as the basis for the substituted diets. Even though the diet is called "high-fat", it also has a considerable amount of carbohydrates (36 energy%), especially in the form of sucrose, thus being closer to the typical obesogenic "Western" diet than other widely used high-fat diets, such as those in which 60% energy is derived from fat. The specification sheet of the high-fat base diet is shown in the Appendix.

3.2 The working heart method and cardiac substrate utilization

Cardiac effects of Calanus oil supplementation were investigated in isolated rat hearts; Functional parameters and myocardial substrate utilization were assessed in the isolated perfused working heart model (Figure 5). In this *ex vivo* model, the heart is removed from its natural milieu and therefore will not reflect the complete *in situ* characteristics of the heart [75]. However, the working heart model has become an important tool for characterizing cardiac phenotype in animal models, allowing assessment of both heart function and metabolism in the same experiment.

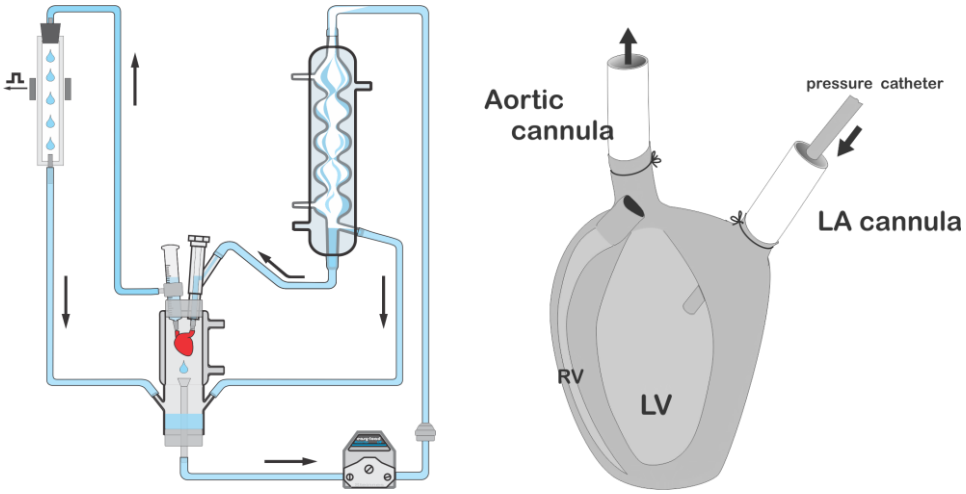

Figure 5. Experimental set-up of the Working Heart Model. Perfusate: Krebs-Henseleit-Bicarbonate buffer contained 200 µmol/L albumin-bound palmitate and 11.1 mmol/L glucose. RV=right ventricle, LV=left ventricle, LA=left atrium

The main substrates used by the heart are fatty acids (FAs) and glucose, and their relative contribution to the energy (ATP) production is regulated by the substrate availability according to the "glucose-fatty acid-cycle", also known as the Randle cycle [76]. When plasma levels of circulating FAs are high, such as in the diabetic or pre-diabetic state, myocardial FA uptake is increased and FAs become the main source of energy for the heart [77 78].

In our experiments, myocardial substrate metabolism was assessed by adding trace amounts of radioactively labeled glucose ([U-^{14}C]-glucose) and palmitate ([9,10-^{3}H]-palmitate) to the perfusion buffer and trapping of their end-products ($^{3}H_2O$ or $^{14}CO_2$).

3.3 Glucose tolerance test

Glucose tolerance was assessed by an intraperitoneal glucose tolerance test (IPGTT).
Oral glucose tolerance tests (OGTTs) are more physiological and, due to increased incretin secretion following elevated enteral glucose concentrations, differences in glucose tolerance between groups might be easier revealed via OGTTs than IPGTTs [79, 80]. However, the IPGTT is still a good, widely used, and not at least easily applied method of assessing glucose tolerance. We also find it less stressful to the animals and chose therefore to carry out IPGTT in both the rat and mouse studies.

3.4 Intra-abdominal fat depots

There is a strong association between intra-abdominal fat (also called "visceral fat") and the risk for various diseases. Therefore, measurements of abdominal fat depots are cornerstones of studies on diet-induced obesity. There are several terms for the different fat depots inside the abdominal cavity of rodents. They are often, though not necessarily correctly, used interchangeably. Our definition of intra-abdominal fat depots in rodents includes the following four depots: the omental, mesenteric, perirenal (pWAT) and perigonadal fat. The latter is called epididymal fat (eWAT) in males and is the most frequently reported fat depot in rodent obesity studies. We measured pWAT and eWAT, two depots that are distinct and easily identified, and can therefore be dissected out and weighed with high precision. While omental and mesenteric fat depots are drained via the portal vein, leading directly into the liver, pWAT and eWAT are drained systemically. According to the "portal theory", increased non-esterified fatty acids (NEFA), and possibly cytokines from visceral fat cause hepatic insulin resistance [81]. Thus it would have been desirable to also have investigated a depot that is drained via the portal vein. However, it has been shown that surgical removal of eWAT and pWAT in rats improves insulin sensitivity [82, 83], indicating an important metabolic role in rodents. Furthermore, reduction of pWAT and mesenteric fat has been shown to be fairly similar in young animals under caloric restriction[84]. Thus we investigated pWAT as a representative of the intra-abdominal fat while at the same time being aware of metabolic differences within intra-abdominal fat depots.

3.5 Liver triacylglycerol content

For the assessment of hepatic steatosis, liver triacylglycerol (TAG) content was measured by the method of Folch et al. [85]. For years, increased TAG content in tissues such as liver and skeletal muscle has been proposed as the cause for insulin resistance. This has been challenged by a number of investigators, and there is now good evidence that high concentrations of diacylglycerols (DAG) or ceramides, rather than TAG, are causally connected to insulin resistance [86]. Nevertheless, when taken as an indicator of fat accumulation, instead of causal evidence, we think that tissue TAG concentration is still a good marker.

3.6 Immunohistological investigations

Identification of macrophages in our study has been done by staining with the antibody F4/80. It is an extracellular antigen (glycosylated proteoglycan) found on murine macrophages [87]. The human equivalent to F4/80 is EMR1.

Macrophages in adipose tissue of obese individuals are known to form so-called crown-like structures (CLS), surrounding dead adipocyte(s) [22]. The presence of CLS was confirmed in our obese animals.

As mentioned in the introduction, macrophages can be divided into the proinflammatory M1- and the anti-inflammatory M2-subpopulation. The F4/80 marker is merely a general macrophage marker, whereas others such as CD11c (M1) or MGL-1 (M2) can distinguish between the two populations [21, 88]. Ideally we would have used one or several of those more specific antibodies. On the other hand, the CLS in our immunohistochemistry sections were very typical, and other groups have identified the macrophages in CLS to be of the M1 population [88]. Therefore, we are confident that the choice of the F4/80 antibody was adequate for our purposes.

3.7 Choice of EPA/DHA preparation

In paper II we compared the Calanus-derived wax ester with ethyl ester-bound EPA and DHA. In commercially available n-3 PUFA products, the majority of long-chain fatty acids is bound in three different chemical forms: triacylglycerols (fish and cod liver oil), phospholipids (Antarctic krill oil) or ethyl esters (EPA + DHA concentrates). Even though a lower bioavailability of ethyl ester-bound PUFAs compared to other preparations such as TAG has been discussed in humans [89], findings are not consistent [90], probably because absorption seems to be dependent on co-administration of fatty meals [91]. In addition, the only prescription form of omega-3 fatty acids for humans is an ethyl ester preparation of EPA + DHA (LOVAZA/OMACOR). Most importantly for our rodent experiments is the fact that Gorreta et al. [92] found a similar bioavailability of EPA and DHA, regardless of whether n-3 PUFAs were given as a TAG-, an ethyl ester- or a wax ester- preparation. Thus, we chose ethyl ester-bound EPA and DHA as a control.

4. SUMMARY OF RESULTS

Paper I: C57Bl/6 mice fed a high-fat diet (45% energy from fat) supplemented with 1.5% (w/w) Calanus oil showed significantly reduced body weight gain, abdominal fat and hepatic steatosis and improved glucose tolerance when compared to mice fed the high-fat diet alone. In adipose tissue, Calanus oil supplementation significantly reduced adipocyte size and it increased mRNA expression of adiponectin. It also significantly reduced macrophage infiltration, accompanied by reduced mRNA expression of pro-inflammatory cytokines (TNFα, IL-6 and MCP-1). The effects of Calanus oil were not only preventive, but also therapeutic, as the oil proved to be beneficial, regardless of whether supplementation was started before or after the onset of obesity and glucose intolerance.

Paper II: C57Bl/6 mice received a high-fat diet (HFD; 45% energy from fat) and were supplemented with either 1% (w/w) wax ester (WE) or 0.2% (w/w) EPA and DHA ethyl ester (E/D) after obesity and glucose intolerance was established. Compared to mice fed HFD without supplementation, WE significantly reduced body weight gain, abdominal fat and hepatic steatosis while improving glucose tolerance and aerobic capacity. In abdominal fat depots, macrophage infiltration was significantly reduced, mRNA-expression of pro-inflammatory genes (TNFα, IL-6 and MCP-1) downregulated and adiponectin expression up-regulated. By comparison, E/D did not significantly affect any of the obesity parameters (body weight gain, abdominal fat or heatic steatosis) or mRNA-expression of adiponectin. It did, however, suppress the expression of pro-inflammatory genes and improved glucose tolerance, although not to the same extent as WE.

Paper III: Analysis of the biochemical composition of Calanus oil revealed a very high percentage (>80%) of fatty acids bound as wax esters. The wax esters consist of primarily C20 and C22 unsaturated fatty alcohols and a variety of long-chain fatty acids. The fatty acid composition showed high amounts of n-3 PUFAs (mainly EPA, DHA and SDA) and MUFAs (mainly gondoic and cetoleic acid) present in the oil. Toxicologic analysis of Calanus oil demonstrates that all values for heavy metals, pesticides, organic and non-organic impurities either fall well below the maximum limits or at levels which do not pose a toxicological concern. Dietary supplementation of obese rats with up to 1.5% (w/w) Calanus oil seemed

safe with no apparent impairment of physical status or physiological parameters, including blood parameters, organ weights and cardiac function. Cardiac metabolism was not affected by Calanus oil supplementation.

5. DISCUSSION

5.1 General discussion

This thesis shows that oil from the marine zooplankton *Calanus finmarchicus* has a unique composition of fatty acids as well as low toxicity levels. Oral supplementation with up to 1.5 % (w/w) is well tolerated and does not seem to negatively affect physiological functions in rodents. On the contrary, in a concentration of 1.5% (w/w) it seems to have beneficial effects in diet-induced obese animals, as shown by:

1) Reduced body weight gain
2) Reduced intra-abdominal fat tissue mass
3) Reduced inflammation in intra-abdominal fat tissue
4) Reduced hepatic steatosis
5) Improved glucose tolerance

Supplementation with 1% Calanus oil-derived wax esters (i.e. fatty acids bound to long chain fatty alcohols), showed almost identical results. However, supplementation with purified EPA/DHA ethyl esters in a concentration similar to the total n-3 PUFA content of the Calanus oil/wax ester diet, did not show significant effects on parameters listed above, except for the anti-inflammatory effects. Figure 6 is a simplified figure showing the main findings and differences between the supplements used in the present study.

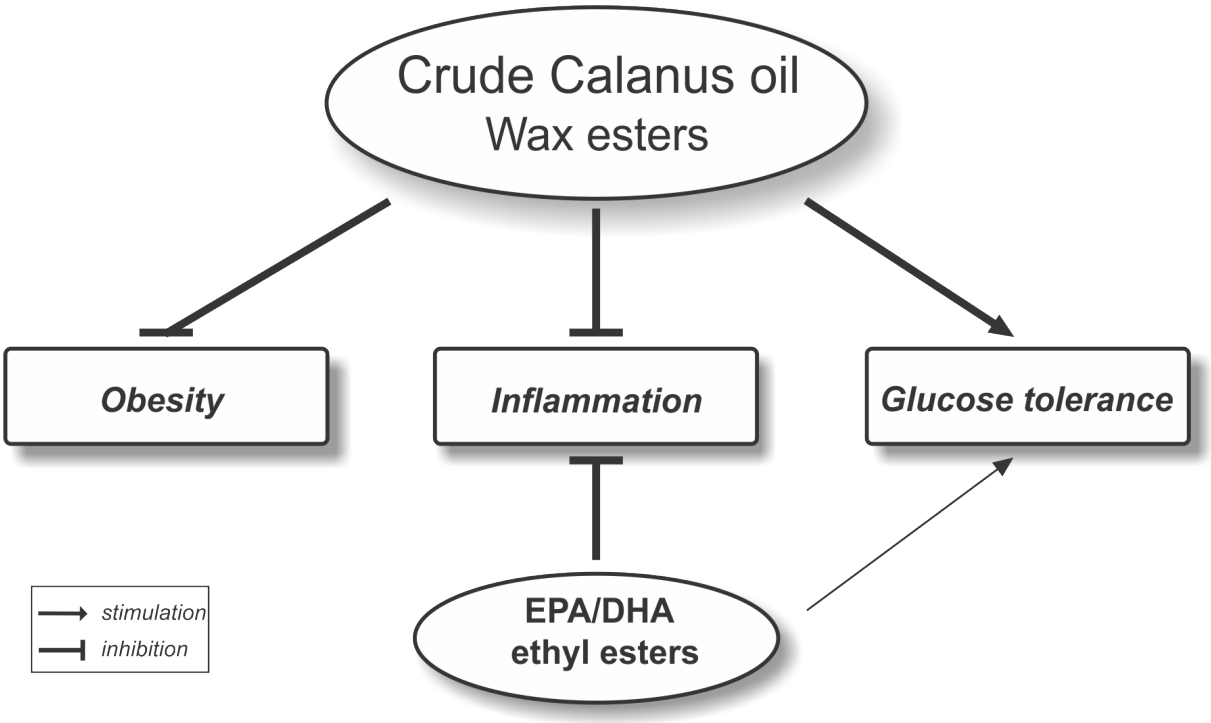

Figure 6. Schematic overview over findings of this thesis. Effects of Calanus oil, wax esters and EPA/DHA ethyl esters on inflammation, glucose tolerance and obesity (including increased body weight, abdominal fat and hepatic fat content). This thesis shows that, while EPA/DHA supplementation mainly influences inflammation, supplementation with both wax esters and crude Calanus oil has beneficial effects on all parameters measured.

As shown in the above illustration, the mutual interface of crude Calanus oil, purified wax esters and purified EPA/DHA ethyl esters was reduced inflammation, while glucose tolerance was only slightly affected and there was no influence on obesity parameters under EPA/DHA ethyl ester supplementation. Therefore, it is evident that some factor, other than n-3 PUFAs, is contributing to the beneficial effect of Calanus oil, especially to the reduction of body weight, abdominal fat and hepatic steatosis, summarized as "obesity" in the figure.

The main difference to other commonly used marine oils is the high amount of wax ester-bound fatty acids. Also, Calanus oil is rich in astaxanthin and contains considerable amounts of phytosterols. In animal studies, phytosterols and/or astaxanthin have been shown to have similar effects as Calanus oil on some of the parameters measured in our study; namely reduced body weight gain and abdominal fat mass [93-96], reduced hepatic steatosis [95 96], reduced insulin levels and improved glucose metabolism [97-99]. On the one hand, it is important to note that the substances in those studies were applied in much higher doses than in our Calanus diet. Most importantly, however, is that we found almost identical results when supplementing the purified wax ester fraction of Calanus oil. This extract is a pure fatty acid/fatty-alcohol solution, devoid of any other compounds. Thus, a pivotal contribution of

phytosterols or astaxanthin to the beneficial effects of Calanus oil is unlikely. The following discussion will therefore focus on the potential role of the wax esters and the fatty acids bound within.

5.2 Body weight, abdominal obesity and hepatic steatosis

Compared to the un-supplemented animals on a high-fat diet, both Calanus oil-fed and wax ester-fed animals were clearly less obese, as indicated by significantly reduced body weight gain, abdominal fat and hepatic steatosis. Obesity is the consequence of an imbalance of anabolic and catabolic processes. Mechanisms of weight/fat reduction include decreased energy intake, suppressed lipogenesis, combined with increased lipolysis and fatty acid oxidation, lipid catabolism in the small intestine, reduced fat absorption and/or increased gastric emptying, all of which previously have been reported for n-3 PUFA supplementation in animals [100-105]. In this thesis however, anti-obese effects in the EPA/DHA ethyl ester group were not observed. This may be related to i) the fact that we started the treatment after the establishment of obesity, while most studies showing beneficial effects of n-3 PUFAs use a preventive rather than a therapeutic approach [101, 106-108], and/or ii) the much lower concentration of EPA/DHA given in our diet as compared to earlier studies. Body weight reduction by n-3 PUFAs seems dose-dependent [106]and the EPA/DHA concentration in our studies was about 7-35 times lower than in other studies [101, 106-108].
Another fatty acid group which could possibly be responsible for anti-obesity effects are MUFAs, which are highly abundant in Calanus oil and its wax esters. Diets rich in plant-derived MUFAs show inconsistent data in humans, but overall seem to have an either neutral or reducing effect on body weight gain [55] and they have also been shown to decrease liver fat and increase lipolysis [109-111]. Interestingly, in a recent study on obese and diabetic mice, supplementing a MUFA-rich marine oil, leads to similar results as in our study [57], indicating a role of MUFAs in obesity and obesity-related disorders.

5.3 Inflammation and glucose metabolism

We showed that Calanus oil and its wax esters attenuated a local inflammatory response in abdominal adipose tissue, including reduced adipocyte size, inflammatory gene expression and macrophage infiltration in abdominal fat tissue. In addition, adiponectin gene expression

was increased, glucose and insulin levels were lowered and glucose tolerance improved. As local pro-inflammatory mediators can create a systemic inflammation that adversely affects metabolic function [15], improved glucose tolerance in Calanus oil/wax ester-supplemented obese mice could, at least in part, be explained in terms of the simultaneous reduction of the inflammatory status.

Pro-inflammatory cytokines can also downregulate the adipocyte-derived hormone adiponectin [112], known for its insulin-sensitizing properties. Consequently, by a reduction in inflammation, adiponectin expression can be recovered, which in turn could improve glucose tolerance. Interestingly, though, despite the adipocyte-reducing and anti-inflammatory effect of the EPA/DHA diet, adiponectin expression was not altered and glucose tolerance was less improved in EPA/DHA-fed animals compared to the wax ester-fed animals. In contrast to our findings, adiponectin has been shown to be upregulated by n-3 PUFAs [113-115]. The reason for this discrepancy could be due to the extremely low concentration of EPA/DHA in our diet as compared to that of previous studies [113-115], or it may be related to the the lack of obesity-reduction, as weight loss results in an upregulation of adiponectin [112].

Also, MUFAs or MUFA-rich diets have been shown to upregulate adiponectin and improve glucose metabolism [116, 117 56, 118]. Yang et al. has done feeding studies with MUFA-rich fish-oils from saury (*Colocabis saira*) [57] and pollock (*Theragra chalcogramma*) [119], showing results similar to ours, such as reduction of abdominal fat [57], reduced hepatic steatosis [119] as well as improvement in glucose homeostasis[57] and increased adiponectin[57, 119]. These oils are rich in gondoic and cetoleic acid, but their content of EPA and DHA is also relatively high. A beneficial additive effect or synergism of MUFAs and PUFAs in those oils is highly plausible, and this could also be suspected for Calanus oil and its wax esters.

Unfortunately, Yang et al.[57, 119] did not show the fat classes of the oils used. However, Ota et al. [120] showed that, despite feeding mainly on copepod species, the dominating fat classes of Pacific saury are TAG, NEFA and phospholipids, while the wax ester content is minimal. In Calanus oil, on the other hand, wax ester-bound FA comprise over 80% of the oil.

It should again be noted that the oils in the studies of Yang et al.[57, 119] were applied at a much higher dose than the Calanus oil in the present feeding studies. So the question remains how we could obtain similar effects with a much lower concentration.

Recently, products from Antarctic krill *(Euphasia superba)*, containing high amounts of EPA and DHA, but also anti-oxidants like astaxanthin, have emerged as a possibly health-promoting food supplement. The majority of fatty acids in krill is bound in phospholipids,

which has been suggested to be the reason for the apparently superior effect of krill compared to fish-oil products [121, 122]. Interestingly, in recent studies, krill oil supplementation in obese rodents showed not only similar results to ours (reduction of glycemia [123, 124], inflammation [123, 125]and hepatic lipids[123-125]), but they were also achieved with relatively low doses. Concentrations of EPA and DHA were only about 2-3 times higher than in our diets, hence well below concentrations used in other fish-oil studies.
Taken together, even though a synergistic effect of MUFAs and PUFAs is highly possible in the case of Calanus oil/wax ester-mediated health effects, the type of fatty acid binding within the oil could also be of importance.

5.4. Wax esters

Although part of the human diet for centuries [126], little has been investigated in terms of beneficial health effects of wax esters; A cholesterol-lowering effect is suggested for plant-derived wax esters [126], whereas marine wax esters have gained a rather negative reputation due to outbreaks of so-called "keriorrhea" –an oily discharge from the rectum following ingestion of wax ester-rich fish [127, 128]. This phenomenon lead to the assumption that mammals cannot digest wax ester. However, there is good evidence that mammals can digest wax esters, at least to a certain degree [92, 129-131]. At the same time, there are indications of a delayed digestion of wax esters with hydrolysis continuing beyond the small intestine [132].

This is very interesting in the light of findings by Morishita et al. [133], showing that after a glucose challenge, secretion of glucagon-like peptide-1 (GLP-1), an insulinotropic incretin, was increased by delivery of DHA and EPA locally in the colon, but not in the stomach or proximal jejunum. The increased GLP-1 secretion resulted in a significant plasma glucose reduction [133]. GLP-1 is produced by L-cells in the distal intestine (ileum and colon). Of note, L-cells have been reported to be co-located with GPR120, a receptor for n-3 PUFA, which has been shown to mediate potent anti-inflammatory and insulin-sensitizing actions, and whose dysfunction might be an underlying factor for diet-induced obesity [41, 42]. It is therefore tempting to speculate that a delayed release of wax ester-bound n-3 PUFAs could have activated intestinal GPR120 and thereby contributed to the beneficial metabolic effects of wax esters observed in the present study.

Also, hydrolysis of wax esters yields fatty acids and fatty alcohols in equal parts. Long-chain alcohols per se have been shown to increase physical performance [134, 135] and exert anti-inflammatory effects [136]. We have access to preliminary data from studies with Calanus oil showing that fatty alcohols are discharged with the feces (Pedersen et al., Norwegian College of Fishery Science, UiT The Arctic University of Norway, unpublished results). However, as we do not have quantitative data on the percentage of fatty alcohol excretion, the role of fatty alcohols per se, regarding the beneficial effects of Calanus oil, is uncertain and requires further investigation.

5.5. The search for "the magic bullet"

In this discussion we have been focusing on the potential role of mono- and polyunsaturated fatty acids on beneficial health effects of Calanus oil. The question is whether it is reasonable trying to single out one individual active component in a natural product or diet, "dissecting" it into singular potentially beneficial components. One has to keep in mind that the result of a treatment is often the effect of several different components acting together. The single components could even have opposite effects, but it is the sum of all that accounts for the final effect.

There are many examples that beneficial health effects of specific diets or natural products cannot solely be attributed to one single component. For example: Despite a complex micro- and macronutrient composition of the famous "Mediterranean diet", authors tend to pinpoint MUFAs as the beneficial factor in the Mediterranean diet. This results from the widespread use of MUFA-rich olive oil. However, beneficial effects of olive oil cannot be reduced to the high MUFA content alone, but may be dependent on other components such as polyphenols[137]. Likewise, studies with marine oils generally ascribe their beneficial effects to EPA and DHA, although the composition of marine oils is often very complex. Also, fish in its natural form can have beneficial effects over fish-oil products [138].

Thus, in all natural products, it is more likely that several components act in an additive or synergistic way rather than one being "the magic bullet". As Hansen et al. already suggested almost 20 years ago, the beneficial effects of the Inuit diet it is probably a combination of both n-3 PUFAs and MUFAs, but also antioxidants [139]. The same is likely to be true for

Calanus oil. Even if it was evident from our experiments that the active compounds of Calanus oil are confined to the wax ester fraction, non-fatty acid components such as the anti-oxidant astaxanthin are probably contributing, for example by limiting oxidation of fatty acids and thus increasing its stability and shelf life. Also, apart from a possible favourable action related to activation of PUFA-recognizing receptors in the distal part of the intestine, the binding of fatty acids to fatty alcohols in the form of wax esters is probably an additional protective factor; Due to their solid or semi-solid form, wax esters present a more stable (less prone to oxidation) and thus more palatable form of n-3 PUFA supply [92].

Although it is of commercial interest (functional food industry) to identify the active component(s) of Calanus oil, research to reveal the underlying physiological mechanisms by which it exerts its beneficial effects should be given priority.

6. SUMMARY AND FUTURE IMPLICATIONS

6.1 Summary

Calanus oil as a dietary supplement is well tolerated and exerts beneficial effects against obesity and obesity-related disorders in rodents. Although the biologically active components are not identified, they seem to be confined to the main lipid fraction (wax esters) of the oil. Most likely, its beneficial effects are dependent on an interaction between different MUFAs and PUFAs, as well as on a rather slow digestion and uptake into the circulation at the distal part of the intestine. Calanus oil seems to be a good alternative to other n-3 PUFA containing supplements, but has the advantage of being naturally low in environmental contaminants and effective in very small amounts.

6.2 Future implications

Even though Calanus oil has shown beneficial health effects in obese rodents, our findings do not provide specific insight into the mechanistic action of the oil. The beneficial effects, however, seem to be related to the reduction of intra-abdominal adipose tissue, as well as reduced adipose tissue inflammation. Future experiments measuring whole body energy expenditure and motor activity are planned in our laboratory in order to explain the fat reducing effect of Calanus oil. Finally, it remains to be shown whether the effects of Calanus oil, as demonstrated in rodents, can be extrapolated to humans.

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APPENDIX

DIO Rodent Purified Diet w/45% Energy From Fat - Red 58V8

DESCRIPTION

Diet Induced Obesity (DIO) Rodent Diet with 45% Energy From Fat, Dyed Red is a Purified Diet based on AIN-76A Semi-Purified Diet, Rat or Mouse 5800-B. See Van Heek et al., J. Clin. Invest. 99:385-390, 1997, for initial use of this formula.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
Meal	1810729
Meal, Irradiated	1810730
1/2" Pellet, Irradiated	55629
1/2" Pellet	58125

**Other Forms Available By Request*

INGREDIENTS

Casein - Vitamin Free	23.3060
Lard	20.6840
Sucrose	20.1360
Maltodextrin	11.6530
Dextrin	8.4830
Powdered Cellulose	5.8270
Soybean Oil	2.9130
Potassium Citrate, Tribasic Monohydrate	1.9230
Dicalcium Phosphate	1.5150
DIO Mineral Mix	1.1650
AIN-76A Vitamin Mix	1.1650
Calcium Carbonate	0.6410
L-Cystine	0.3500
Choline Bitartrate	0.2330
Red Dye	0.0060

Part of the TestDiet® "Blue-Pink-Yellow" DIO Series ("van Heek" Series)

DIO Rodent Purified Diet w/10% Energy From Fat - Blue
- 1/2" Pellet - Catalog # 58126 (58Y1)
- 1/2" Pellet, Irradiated - Catalog # 56833 (58Y1)
- Meal - Catalog # 1810473 (58Y1)

DIO Rodent Purified Diet w/10% Energy From Fat - Yellow
- 1/2" Pellet - Catalog # 58124 (58Y2)
- Meal - Catalog # 56834 (58Y2)

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store properly upon receipt. For laboratrory animal use only, not for human consumption.

NUTRITIONAL PROFILE [1]

Protein, %	***21.3***
Arginine, %	0.81
Histidine, %	0.60
Isoleucine, %	1.12
Leucine, %	2.02
Lysine, %	1.69
Methionine, %	0.60
Cystine, %	0.44
Phenylalanine, %	1.12
Tyrosine, %	1.18
Threonine, %	0.90
Tryptophan, %	0.26
Valine, %	1.33
Alanine, %	0.64
Aspartic Acid, %	1.50
Glutamic Acid, %	4.76
Glycine, %	0.45
Proline, %	2.75
Serine, %	1.29
Taurine, %	0.00
Fat, %	***23.6***
Cholesterol, ppm	196
Linoleic Acid, %	3.48
Linolenic Acid, %	0.32
Arachidonic Acid, %	0.04
Omega-3 Fatty Acids, %	0.32
Total Saturated Fatty Acids, %	9.05
Total Monounsaturated Fatty Acids, %	9.32
Fiber (max), %	***5.8***
Carbohydrates, %	***41.2***
Energy (kcal/g) [2]	***4.65***

From:	kcal	%
Protein	0.850	18.3
Fat (ether extract)	2.124	45.7
Carbohydrates	1.649	35.5

Minerals	
Calcium, %	0.70
Phosphorus, %	0.53
Phosphorus (available), %	0.53
Potassium, %	0.70
Magnesium, %	0.06
Sodium, %	0.12
Chlorine, %	0.18
Fluorine, ppm	1.1
Iron, ppm	54
Zinc, ppm	41
Manganese, ppm	68
Copper, ppm	7.0
Cobalt, ppm	0.0
Iodine, ppm	0.24
Chromium, ppm	2.3
Molybdenum, ppm	1.90
Selenium, ppm	0.19

Vitamins	
Vitamin A, IU/g	4.7
Vitamin D-3 (added), IU/g	1.2
Vitamin E, IU/kg	60.6
Vitamin K (as menadione), ppm	0.58
Thiamin Hydrochloride, ppm	7.0
Riboflavin, ppm	7.0
Niacin, ppm	35
Pantothenic Acid, ppm	17
Folic Acid, ppm	2.3
Pyridoxine, ppm	6.7
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	12
Choline Chloride, ppm	1,165
Ascorbic Acid, ppm	0.0

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

4/25/2006

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