UiT The Arctic University of Norway

The Norwegian College of Fishery Science
Department of Medical Biology

Calanus oil and its constituents as a therapeutic approach to target obesity-induced metabolic distortions

Pauke Carlijn Schots

A dissertation for the degree of Philosophiae Doctor – June 2023

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List of papers and author contributions

Paper I:
Schots, P. C., Pedersen, A. M., Eilertsen, K.-E., Olsen, R. L., & Larsen, T. S. (2020). **Possible Health Effects of a Wax Ester Rich Marine Oil** [Review]. *Frontiers in Pharmacology*, *11*(961). https://doi.org/10.3389/fphar.2020.00961

Paper II
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* Both authors contributed equally to this work and share first authorship.

Paper III
Schots, P. C., Edvinsen, G. K., & Olsen, R. L. (2023). **A simple method to isolate fatty acids and fatty alcohols from wax esters in a wax-ester rich marine oil** *PLOS ONE*, *18*(5), e0285751. https://doi.org/10.1371/journal.pone.0285751

Paper IV
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Co-Author contributions

	Paper I	**Paper II**	**Paper III**	**Paper IV**
Concept and idea	PCS, AMP, TSL	JM, TSL	RLO	PCS, WL, TSL, EA
Study design and methods		PCS, KMJ, JM, TSL	PCS, GKE, RLO	PCS, WL, EA, TL
Data gathering and interpretation	PCS, AMP, KEE, RLO, TSL	PCS, KMJ, JM, AMP TSL	PCS, GKE, RLO	PCS, WL, AS, TSL, EA
Manuscript preparation	PCS, AMP, KEE, RLO, TSL	PCS, KMJ, RLO TSL	PCS, RLO	PCS, TSL, EA

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Research plan – challenges and obstacles

The plan for my PhD project was based on previous results obtained in both the Seafood Science and the Cardiovascular research groups, the two research groups where I was admitted as a PhD candidate in 2017. They had reported beneficial metabolic effects, in particular a reduced deposition of visceral fat, reduced adipose tissue inflammation, and improved whole body glucose tolerance in obese mice where a high-fat diet was supplemented with 1-2% Calanus oil. Calanus oil is a relatively new marine oil, obtained from the crustacean *Calanus finmarchicus*. Unlike other marine oils, Calanus oil is rich in wax esters, which are fatty acids are esterified to fatty alcohols. It contains relatively little eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the well-studied omega-3 fatty acids that are major constituents of most other marine oils, but rather contains a cocktail of different long-chain poly-and monounsaturated fatty acids.

The original plan was to study the impact of Calanus oil supplementation on energy metabolism (oxidation) in energy-consuming tissues (muscle, liver, and kidney) by examining its impact on mitochondrial respiration and associated alterations in gene expression. We also wanted to study potential alterations in mobilization and deposition of fat in response to Calanus oil intake, hoping to explain the anti-obesogenic effect of the oil.

However, in 2017 the animal facility at the Faculty of Health Sciences closed due to renovation and expansion of the facility. Reopening was postponed several times and did not occur until the fall of 2021. This situation had serious negative consequences for many research groups at the faculty, including my own project. To do some useful work in this period, I investigated the impact of Calanus oil on the composition of the gut microbiome in mice, using biological material sampled before closure of the animal facility. In addition, I took part in studies on mitochondrial respiration in various tissues from mice (treated with Calanus oil or a GLP-1 receptor agonist), which was a collaborative initiative between UiT and the University of Barcelona. Unfortunately, during the shipping of our samples from Barcelona to Tromsø for further analyses, the samples got lost, and we received them 3 weeks later. The samples were thawed, and it was therefore not possible to do the extra analyses necessary for a publication within my thesis. After returning from Barcelona, I wrote a review paper about the different components of Calanus oil and how they can control inflammation and reduce metabolic syndrome.

The lack of access to a working animal facility at UiT eventually led us to use *in vitro* cell lines as an experimental model instead of animal models. Based on the hypothesis that adipocyte expansion plays a central role in obesity-induced insulin resistance, we started to work with 3T3-L1 preadipocytes. We exposed the cell to high levels of palmitate (a saturated fatty acid) to mimic obesity, but unlike several reports in the literature, we were not able to induce insulin resistance by this approach. After countless attempts and variations in the protocol, we had to change our experimental model once more. This time we took advantage of experience within the research group and worked with H9c2 cells, which are cardiomyoblasts derived from embryonic rat heart tissue. It had been observed that micromolar concentrations of hydrolysed Calanus oil wax esters protect these cells from palmitate-induced cell death. The task for the final part of my PhD project was to develop a method to separate the fatty acids in hydrolysed Calanus oil wax esters from the fatty alcohols, and to test their individual and combined potential to protect H9c2 cells from palmitate-induced (lipotoxic) cell death.

Summary

Obesity is one of the leading causes to the development of metabolic disorders, such as insulin resistance and type 2 diabetes. Obesity and being overweight are disorders that have reached epidemic proportions, with more than 4 million people dying each year as a consequence of these conditions. Several therapies have been developed to treat obesity and its accompanying consequences, but none have so far proven to be effective and/or safe.

Omega-3 fatty acids, found in seafood and marine oils, have been shown to reduce obesity, lower blood lipid levels, improve the composition of the gut microbiota and reduce the risk for cardiovascular diseases. It is therefore recommended to consume two servings of fish per week or 250 mg/day of EPA and DHA, two omega-3 fatty acid present in seafood. However, the increased demand for omega-3 fatty acids has put pressure on sustainable fisheries, thus there is a need for alternative sources of these marine lipids. The marine crustacean *Calanus finmarchicus* is such an alternative source. Calanus oil is unique compared to other marine oils, in the sense that more than 85% of the lipids are wax esters, a lipid class where fatty acids are bound to fatty alcohols. The oil has relatively low concentrations of EPA and DHA but is rich in stearidonic acid (another omega-3 fatty acid) and long-chain monounsaturated fatty acids. In experimental studies, dietary supplementation with Calanus oil was shown to reduce obesity and obesity-related inflammation, improve glycaemic control, and protect the heart after an ischemic event.

The aim of the current thesis was to get a better understanding of how Calanus oil and its constituents could possibly be used as a therapeutic approach to target obesity-induced metabolic distortions. In paper I we reviewed previously published results regarding the impact of the different fatty acids and fatty alcohols present in Calanus oil on obesity-induced low-grade inflammation. In paper II we found that Calanus oil had the tendency to change the microbial composition in the gut towards a healthier phenotype, although the changes were not statistically significant. Paper III describes the method we developed to separate the fatty acids from the fatty alcohols in hydrolysed Calanus oil wax esters using solid-phase extraction. In paper IV we show that these fatty acids effectively, and in a dose-dependent manner, improved the viability of palmitate exposed cardiac H9c2 cells. The protective effect was associated with increased fatty acid oxidation, which will alleviate lipotoxic stress in the cells. We conclude that Calanus oil and/or its wax ester-derived fatty acids can be used as a therapeutic approach to target obesity-induced metabolic disorders.

Abbreviations

AMPK	Adenosine monophosphate-activated protein kinase
AP-1	Activator protein 1
ATF	Activating transcription factor
CHOP	DNA damage-inducible transcript 3
CPT	Carnitine palmitoyltransferase
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
eIF2α	Eukaryotic translation initiation factor 2α
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FA	Fatty acids
FAC	Calanus oil wax ester-derived fatty acids
FAME	Fatty acid methyl ester
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter
GM	Gut microbiome
GPR	G-protein coupled receptor
HFD	High-fat diet
HSL	Hormone-sensitive lipase
IKK	IkB kinases
IL	Interleukin
IR	Insulin receptor
IRE1	Inositol-requiring enzyme 1
IRS-1	Insulin receptor substrate 1
JNK	c-Jun N-terminal kinases
MUFA	Monounsaturated fatty acid
n-3 PUFA	Omega-3 polyunsaturated fatty acid
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MKK	Mitogen-activated protein kinase kinase
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OTU	Operational taxonomic unit
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PERK	Protein kinase R-like endoplasmic reticulum kinase
PI3K	Phosphoinositide 3-kinases
PKC	Protein kinase C
PP2A	Protein phosphatase 2
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
SDA	Stearidonic acid
SPE	Solid-phase extraction
SREBP	Sterol regulatory element-binding protein
TAG	Triacylglycerol
TAK1	Transforming growth factor-β activated kinase-1
TCA	Tricarboxylic acid cycle
TLC	Thin-layer chromatography
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
UPR	Unfolded protein response

1 Introduction

Obesity is defined as the accumulation of fat to the extend it presents a threat to human health. It is the main risk factor in the development of metabolic diseases, such as insulin resistance and type 2 diabetes, and it shortens life expectancy and increases the risk of cardiovascular diseases (Malik et al., 2020; Malik et al., 2013). In thermodynamic terms, obesity arises only when energy intake exceeds energy expenditure. Therefore, a lifestyle promoting high energy intake and low energy expenditure is believed to be the major factor explaining the current obesity epidemic. In line with this view, the recommendation to combat obesity is still based on the general principles of a healthy lifestyle, especially exercise and dietary interventions. This is, however, not sufficient. The pharmaceutical industry has developed several drugs for weight reduction and long-term weight maintenance, but drugs with proven long-term safety and efficacy are still not available. The importance of finding a cure for obesity is based on the observation that obesity is highly associated with type 2 diabetes and that diabetics have a significantly (2 to 3 times) increased risk of cardiovascular complications (Buse & ACCORD Study Group, 2007; Kannel & McGee, 1979). During the last decades it has become evident that the unfavourable changes in adipose tissue metabolism in obesity can be linked to the aetiology of obesity-related cardiovascular diseases. A thorough understanding of these processes is needed for targeted treatment and prevention of the obesity-related distortions.

1.1 Adipose tissue metabolism in obesity

Adipose tissue used to be regarded as an organ that cushions and insulates the body, and where fatty acids (FA) are stored after food intake and released during periods of fasting (Hajer et al., 2008). The uptake and storage of fat in the postprandial phase is catalysed by insulin, which activates lipoprotein lipase (LPL) and thereby hydrolyses triacylglycerol (TAG) in circulating TAG-rich lipoproteins, such as very low-density lipoproteins and chylomicrons (Sadur & Eckel, 1982; Taskinen, 1987). During periods of fasting, when insulin levels are low, these lipid reserves are mobilized through activation of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), releasing FA from adipose tissue, which are transported via the bloodstream to supply other organs with nutrients for energy production (Nielsen et al., 2014; Zimmermann et al., 2004).

Over the years, however, we have learned that adipose tissue is much more than just a storage of fat. It is an endocrine organ that produces a wide range of hormones and cytokines (adipokines) involved in several processes such as glucose metabolism (e.g. adiponectin), lipid

metabolism (e.g. cholesteryl ester transfer protein), inflammation (e.g. tumor necrosis factor (TNF) and interleukin (IL)-6), coagulation (e.g. PAI-1), blood pressure (e.g. angiotensin) and feeding behaviour (e.g. leptin) (Fasshauer & Blüher, 2015; Hajer et al., 2008).

Healthy adipose tissue contains two transcription factors families that serve key roles in adipose tissue metabolism – peroxisome proliferator-activated receptor (PPAR) and sterol regulatory element-binding proteins (SREBP) (Hajer et al., 2008; Sun et al., 2021; White & Stephens, 2010). The PPAR family consist of subtypes α, β (also called δ) and γ (Berger & Moller, 2002). PPAR-γ is highly expressed in adipose tissue and activates genes involved in preadipocyte differentiation and FA trapping, such as adiponectin, acyl-CoA synthase, LPL, and FA binding and transporting proteins. PPAR-γ activation leads to hyperplasia and hypertrophy during increased lipid storage, resulting in lower plasma lipids levels and reduced TAG accumulation in muscle and other, non-adipose tissue, organs (Hajer et al., 2008; Vidal-Puig et al., 1996). SREBP also activates genes necessary for preadipocyte differentiation, as well as for endogenous lipogenesis (Shimano et al., 1999; Shimomura et al., 1998; White & Stephens, 2010).

1.1.1 Adipocyte hypertrophy

Unhealthy adipose tissue, as in obesity, can be seen as the starting point for many conditions and diseases, such as insulin resistance, type 2 diabetes, cardiovascular diseases, and non-alcoholic fatty liver disease (Figure 1). Obesity, combined with high blood pressure, elevated fasting blood glucose and TAG levels, and low levels of high-density lipoprotein cholesterol is commonly referred to as “metabolic syndrome”. These metabolic factors are strong risk factors for cardiovascular diseases and stroke (Gregor & Hotamisligil, 2011; Hajer et al., 2008). The fat depots throughout the body are not equal in terms of associated health risks. Traditionally, intra-abdominal fat is considered as an unhealthy fat depot and is associated with insulin resistance. It is thought that insulin resistance is mediated by lower adiponectin levels, a greater lipolytic activity and an increased production of inflammatory cytokines. Subcutaneous fat, on the other hand, is considered metabolically protective (Hocking et al., 2013).

Obesity occurs as a result of excess energy intake, relative to energy expenditure. This positive energy balance leads to increased lipid storage and consequently adipocyte expansion. Adipocyte hypertrophy is closely related to angiogenesis (Rupnick et al., 2002). But when adipocytes become too large (around 100-200 μm in diameter) there is a mismatch between adipogenesis and angiogenesis (Brahimi-Horn & Pouysségur, 2007). The diffusion distance for

oxygen (from the blood to the individual fat cells) becomes too large, leading to a state of cellular hypoxia. Hypoxia triggers the expression of hypoxia-inducible factor 1α, which leads to an increased release of FA and pro-inflammatory markers, as well as the recruitment and tissue infiltration of macrophages (discussed in more detail below) (Brahimi-Horn & Pouysségur, 2007; Sun et al., 2011). Over time, this situation leads to dyslipidemia and systemic (low-grade) inflammation (Klop et al., 2013), which impairs metabolism and function in the muscle, liver and the vasculature (Figure 1). Several mechanisms have been proposed to link adipose tissue dysfunction to disrupted (systemic) insulin signalling. In this PhD thesis, I will focus on inflammation and lipotoxicity.

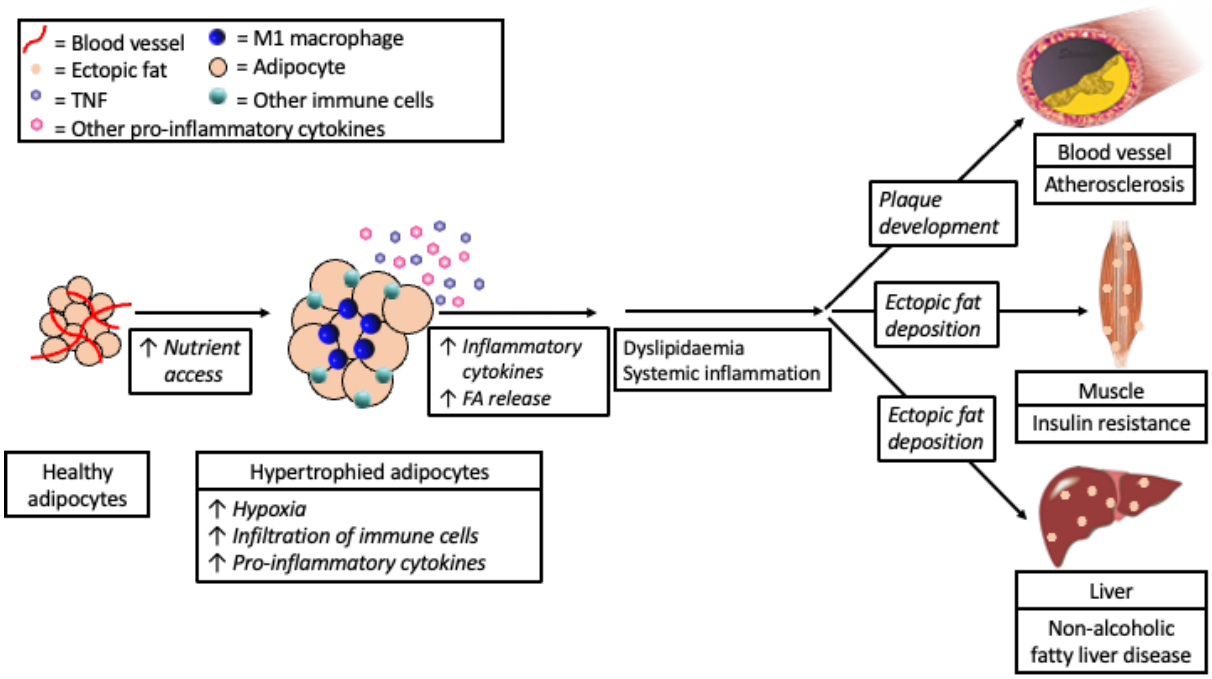

Figure 1. Expansion of adipocytes due to nutrient access leads to dyslipidemia and systemic inflammation, and subsequent cardiovascular disease, insulin resistance, and non-alcoholic fatty liver disease. See the text for detailed explanation and abbreviations. Modified from Schots et al., (2020).

1.1.2 Adipose tissue inflammation

Obesity is commonly associated with changes in the secretory function of adipocytes and macrophages (Bastard et al., 2006). During obesity, adipose tissue M2 macrophages (anti-inflammatory type) shift to M1 macrophages, which secrete pro-inflammatory cytokines such as TNF, IL-6 and IL-1β (Figures 1 and 2) (Gordon & Taylor, 2005; Gregor & Hotamisligil, 2011; Lumeng et al., 2007). Locally, in the adipose depots, obesity leads to a vicious circle of cytokine release by adipocytes and macrophages. Adipose tissue released-saturated FA, as well

as nutritional saturated FA, such as palmitate, can bind to toll-like receptor 4 (TLR4) on M1 macrophages (Figure 2) (McKernan et al., 2020; Suganami et al., 2005; Suganami et al., 2007). The subsequent activation of TLR4 activates transforming growth factor-β activated kinase-1 (TAK1), which plays an important role in the activation of several transcription factors (Wang et al., 2001). TAK1 phosphorylates the IκB kinases (IKK) complex, which in turn leads to the phosphorylation and thereby degradation of IκB. The degradation of IκB releases nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Figure 2), allowing it to translocate from the cytoplasm to the nucleus (Perkins, 2007; Suganami et al., 2005; Suganami et al., 2007). TAK1 also activates the activation protein 1 (AP-1) transcription factor via c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase kinase (MKK) (Figure 2) (Karin et al., 1997; Wang et al., 2001). Activation of these transcription factors leads to the transcription and secretion of pro-inflammatory cytokines by the macrophages. This wave of cytokines together with (dietary) FA bind to TNF receptors (TNFR) and TLR4 on adipocytes, activating the TAK-JNK-NF-κB pathways in these cells. This activation reinforces the production of pro-inflammatory cytokines in adipocytes, thereby completing the vicious circle (Figure 2) (McKernan et al., 2020; Solinas & Karin, 2010).

In healthy adipose tissue, insulin activates the insulin receptor substrate 1 (IRS-1)–phosphatidylinositol 3-kinase (PI3K)–protein kinase B (Akt) pathway by binding to insulin receptors (IR) on the cell surface. The majority of insulin's metabolic effects are mediated via Akt, such as glucose uptake, through the translocation of glucose transporter type 4 (GLUT4) from cytosolic endosomes to the cell membrane, lipid synthesis, by inhibiting FA oxidation and lipolysis, gluconeogenesis, and glycogen synthesis (Figure 2) (Boucher et al., 2014). A chronic inflammatory state in adipose tissue results in local insulin resistance due to the serine phosphorylation of IRS-1 by JNK and IKK, thereby inhibiting the translocation of GLUT4 to the cell membrane and reducing glucose uptake (Figure 2) (Boden, 2011; Hotamisligil et al., 1996; Solinas & Karin, 2010). Furthermore, impaired PI3K-Akt signalling inhibits the synthesis of glycogen and it releases the inhibition of FA oxidation and HSL-mediated lipolysis, leading to an increased release of FA from the adipocytes (Dimitriadis et al., 2011; Huang et al., 2018; Morigny et al., 2016; Nielsen et al., 2014). In addition, JNK blocks the activation of PPAR-γ, and thereby decreases the production of adiponectin (Maeda et al., 2001; Yadav et al., 2013). Adiponectin plays an important role in glucose and FA metabolism, such as stimulating the storage of fat in subcutaneous adipose tissue, and a reduced expression can therefore have detrimental whole-body consequences (Trujillo & Scherer, 2005).

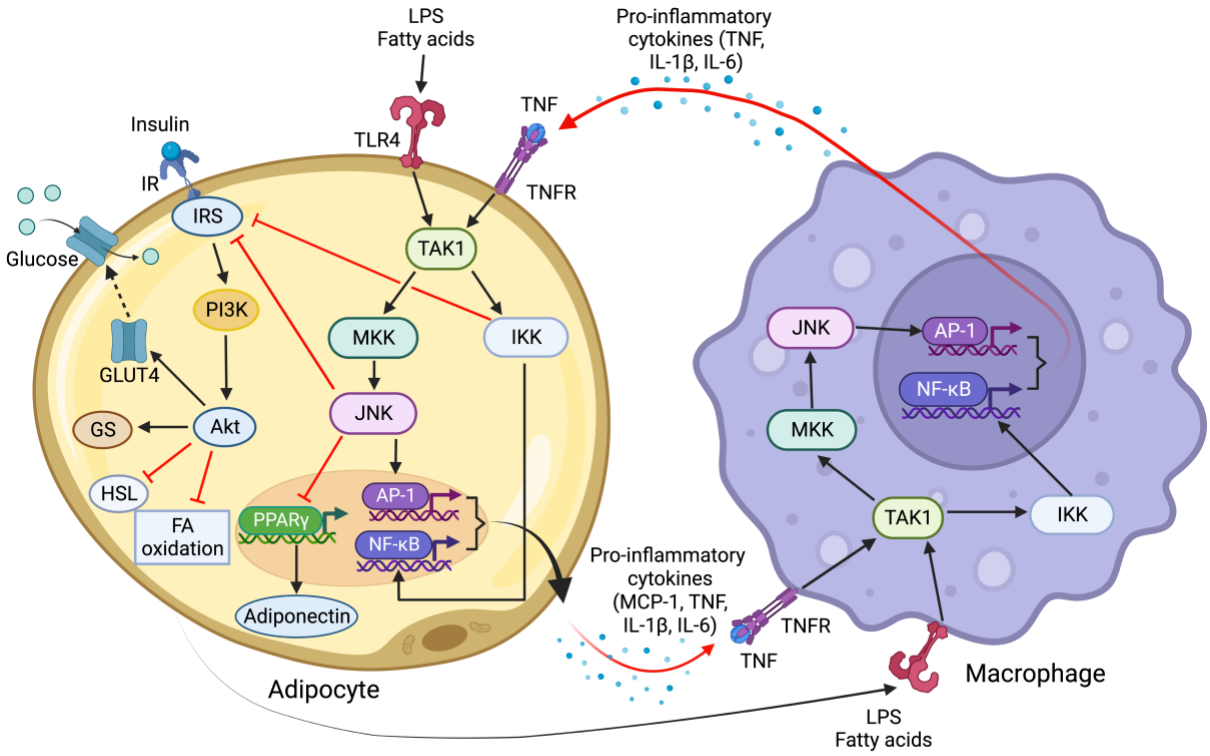

Figure 2. A vicious circle of pro-inflammatory cytokines released by macrophages and adipocytes that eventually leads to insulin resistance in adipose tissue. GS, glycogen synthase; MCP-1, monocyte chemoattractant protein-1. See the text for detailed explanation and other abbreviations. Created in BioRender. Inspired by Hajer et al., (2008).

Obese adipose tissue releases FA and pro-inflammatory cytokines to the blood, giving rise to systemic low-grade inflammation and hypertriglyceridemia, which can cause insulin resistance in peripheral, insulin-sensitive tissues (Boden, 2008; De Luca & Olefsky, 2008). The precise sequence of events and the connections between inflammation in these organs and decreased insulin signalling in (skeletal and cardiac) myocytes is still not fully understood, although ectopic fat appears to play an important role (Shulman, 2014).

1.1.3 Ectopic fat deposition

Ectopic fat is defined as the deposition of TAG within cells of non-adipose tissues that normally contain only small amounts of fat, e.g. heart, liver, skeletal muscle, kidney, pancreas and blood vessels (Shulman, 2014). When enlarged, these fat depots become infiltrated with immune cells that, as explained above, can interfere with insulin signalling through the secretion of pro-inflammatory cytokines (Wu & Ballantyne, 2017). Several mechanisms have been proposed to explain the tendency to deposit adipose tissue in ectopic sites or depots, rather than in adipose, depots. One hypothesis suggests that in states of positive energy balance, excess FA are initially

stored subcutaneously, but once the capacity of this depot is reached, storage shifts to intra-abdominal fat depots followed by ectopic sites (Després et al., 2008; Heilbronn et al., 2004). Insulin resistance, and associated metabolic disturbances, are linked to ectopic fat deposition in skeletal muscle and liver, and increased fat cell size is correlated with insulin resistance and the development of type 2 diabetes (Heilbronn et al., 2004; Larson-Meyer et al., 2006; Lundgren et al., 2007; Wu & Ballantyne, 2017). It is believed that ectopic fat deposition is closely associated to cardiometabolic risk, due to the influence it has on lipid metabolism and promoting endoplasmic reticulum (ER) stress, oxidative stress, and changing the composition and function of cellular membranes (Ferrara et al., 2019). In addition, the deposition of ectopic fat in the heart has been associated with atrial fibrillation and ventricular dysfunction, while perivascular adipose tissue is associated with atherosclerosis and hypertension (Ferrara et al., 2019).

1.2 Metabolism and lipotoxicity in the heart

The heart is one of the most vital organs in our body, whose main purpose is to pump blood to supply other organs and tissues with oxygen and nutrients. The energy demand for supporting cardiac contractile function is high and depends on a continuous production of ATP. The majority (95%) of cardiac ATP is produced via oxidative phosphorylation in the mitochondria, making the heart the most oxygen consuming organ in our body relative to its weight. The remaining 5% of ATP is derived through glycolysis (Lopaschuk et al., 2021). The heart is referred to as an omnivore (Kelley & Mandarino, 2000; Taegtmeyer et al., 2004) and can oxidize a variety of substrates, including FA, glucose, lactate, pyruvate, ketone bodies and amino acids, but with glucose and FA being the most important substrates (Jaswal et al., 2011). The relative contribution of FA and glucose to the energy production depend on the plasma substrate supply, as well as the level of cardiac work (Lopaschuk et al., 2010). During resting conditions, a heathy heart derives 50-60% of its energy requirement from FA oxidation, 30-40% from glucose metabolism and the remaining 10% from lactate (Stanley et al., 2005).

1.2.1 Glucose and fatty acid utilization

Glucose is taken up from the plasma by cardiomyocytes via GLUT4 transport proteins (and to a lesser extend via GLUT1). In addition, endogenous glucose in the form of glycogen can contribute to myocardial energy production. The translocation of GLUT4 from the cytosol to the sarcolemma is stimulated by insulin in a similar fashion as described above in adipose

tissue, and via muscle contraction through adenosine monophosphate-activated protein kinase (AMPK) (Stanley et al., 2005). Glucose is broken down to pyruvate in glycolysis and further decarboxylated to acetyl-CoA by the pyruvate dehydrogenase (PDH) complex. Acetyl-CoA is metabolized in the tricarboxylic acid (TCA) cycle in the mitochondria (Figure 3) (Patel et al., 2014).

LPL hydrolyses circulating TAG, and the liberated FA (as well as albumin-bound FA from adipose tissue) enter the cardiomyocytes via diffusion through the plasma membrane or via specific fatty acid transporting proteins, such as CD36 (Glatz & Luiken, 2020; Stanley et al., 2005). Once across the membrane, FA bind to fatty acid binding proteins and are subsequently converted to fatty acyl-CoA by fatty acyl-CoA synthase, thereby trapping FA inside the cell (Stanley et al., 2005). These FA are broken down to acetyl-CoA units through β-oxidation in the mitochondria, and further metabolized in the TCA cycle (Figure 3) (Schulz, 1991). The metabolism of acetyl-CoA, both from FA and glucose, in the TCA cycle generates reducing equivalents (NADH and FADH2), which are fed into the electron transport chain and produce ATP in the process of oxidative phosphorylation (Hatefi, 1985).

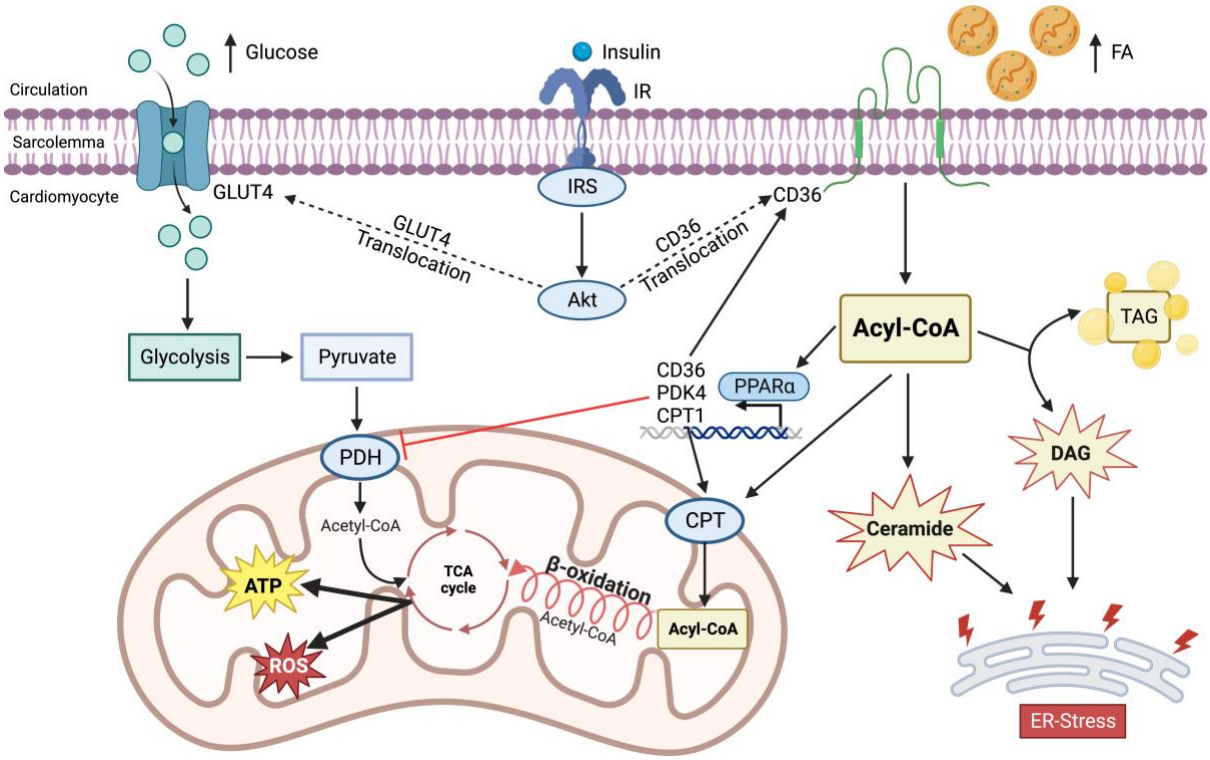

Figure 3. Substrate utilization in heart, and loss of metabolic flexibility due to chronic lipid overload and lipotoxicity. See the text for detailed explanation and abbreviations. Created in BioRender. Inspired by Guzzardi & Iozzo (2011).

The normal heart is characterized by a high metabolic flexibility, to ensure that metabolic supply and demand are optimally matched. Thus, cardiac metabolism of glucose and FA is tightly intertwined and regulated both at the level of substrate uptake and mitochondrial oxidation (Figure 3) (Glatz et al., 2006). As described above, the uptake of FA and glucose is mediated to a large extend by CD36 and GLUT4, respectively. Both transporters are located in endosomes in the cytosol and translocate to the sarcolemma upon stimulation by either insulin or AMPK (Cushman et al., 1998; Glatz et al., 2010; Saltiel & Kahn, 2001). The uptake of FA and glucose can be activated at the same time, although some studies have shown that cells can be selective in the recruitment of either CD36 or GLUT4 to the sarcolemma, thereby facilitating uptake of only one of the substrates (Glatz et al., 2006). The uptake of FA, via the transcription of CD36, also depends on the activation of PPARα, while PPARα itself is again activated by acyl-CoA (Figure 3) (Gilde et al., 2003). PPARα is also involved in the regulation of glucose and FA metabolism on the mitochondrial level, by regulating the expression of several enzymes/transporters, such as pyruvate dehydrogenase kinase 4 (PDK4) (an inhibitor of PDH), and carnitine palmitoyltransferase 1 (CPT1) (Figure 3) (Gilde et al., 2003). FA oxidation also reduces glucose metabolism through increased citrate levels, which inhibit phosphofructokinase 2, an enzyme involved in glycolysis (Chess & Stanley, 2008). The inhibitory effect of FA oxidation on glucose oxidation was first described by Philip Randle in 1963 and is referred to as the Randle cycle (Randle et al., 1963). It describes the shift between dominating glucose and FA oxidation, which take place during acute changes in substate supply.

1.2.2 Altered substrate utilization during insulin resistance and lipotoxicity in obesity

The cardiac muscle in obese and insulin resistant patients is described to be metabolically less flexible in adapting fuel preference, and becomes heavily dependent on FA for energy production (Larsen & Aasum, 2008; Smith et al., 2018). The rate of FA uptake by cardiomyocytes is dependent on the plasma FA concentration, which in turn is determined by the release of FA from adipose tissue (Nielsen et al., 2014). Under pathological conditions, such as chronic lipid overload and insulin resistance, the FA uptake via CD36 has shown to play a particular important role (Glatz & Luiken, 2018). Under these conditions the translocation of CD36 and GLUT4 to the sarcolemma are distinctly different, GLUT4 is trapped within the endosomes, while CD36 is channelled to the sarcolemma, (Luiken et al., 2020), and accounts for 40-60% of the FA uptake (Guzzardi & Iozzo, 2011).

During metabolic homeostasis about 80% of acyl-CoA are oxidized by the mitochondria and the remaining 20% enter the intramyocardial TAG pool to serve as an endogenous source of FA (Lopaschuk et al., 2010). However, in a state of obesity-induced dyslipidemia and hypertriglyceridemia, when the FA supply is elevated, and the FA uptake outpaces the cells energy needs (i.e. FA oxidation), the excessive FA supply leads to the formation of ectopic fat and toxic lipid intermediates, such as, diacylglycerol (DAG), ceramides, and acetylcarnitines (Lopaschuk et al., 2010). It is during this imbalance between FA uptake and FA oxidation, and when the FA storage capacity is exceeded, that lipotoxicity occurs. Lipotoxicity is defined as the accumulation of unoxidized lipids in non-adipose tissue that lead to cellular dysfunction, and eventually cell death, and it is closely related to obesity, type 2 diabetes, and other metabolic disorders (Lee et al., 1994; Schaffer, 2003)

It was suggested by Shulman (2000) that while the Randle cycle describes changes in fatty acid and glucose oxidation during acute changes in substrate supply, it does not explain how a chronic lipid overload leads to insulin resistance. Shulman (2000) proposed a mechanism by which FA-induced insulin resistance occurs through the formation of DAG and ceramides, that function as second messenger molecules and inhibit the insulin signalling pathway through activation of protein kinase C (PKC). This mechanism was later confirmed in experiments on human muscle cells and muscle cell cultures (Blachnio-Zabielska et al., 2016; Chavez et al., 2003; Chavez & Summers, 2003; Macrae et al., 2013; Pickersgill et al., 2007). PKC is involved in various signal transduction pathways, such as the IKK, JNK, and PI3K-Akt (Figure 4). Ceramides, which are produced from saturated FA (specifically palmitate), also activate protein phosphatase 2 (PP2A), which inhibits the PI3K-Akt signalling pathway (Figure 4) (Holland et al., 2007; Macrae et al., 2013; Samuel & Shulman, 2012; Zhang et al., 2012).

In addition to unoxidized lipids, FA that do enter the mitochondria can also contribute to lipotoxicity and lipid-induced insulin resistance. When the FA uptake exceeds the energy needs of the cell, an imbalance between FA oxidation and long-chain acylcarnitine synthesis occurs (also called incomplete FA oxidation), leading to the accumulation of acylcarnitine molecules. This accumulation can induce inflammation, oxidative stress, and insulin resistance by, in part, activation of NF-kB. In addition, increased plasma acylcarnitine levels have been associated with type 2 diabetes (Adams et al., 2009; Aguer et al., 2015). Furthermore, the accumulation of ectopic fat with its infiltrated immune cells contributes to insulin resistance through the secretion of pro-inflammatory cytokines and FA (Figure 4), initiating a similar vicious circle as described in Figure 2 (Wu & Ballantyne, 2017)

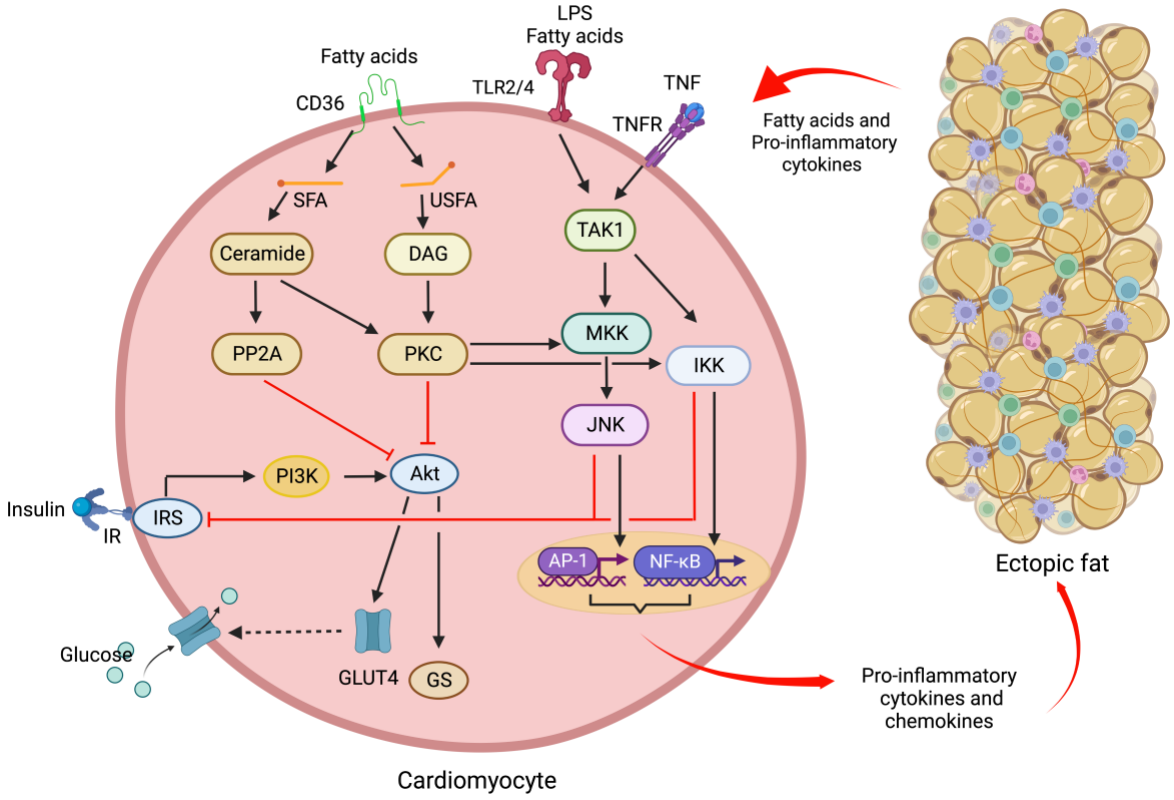

Figure 4. Ectopic fat, with infiltrated immune cells, and the formation of toxic lipid intermediates in cardiomyocytes can lead to insulin resistance. GS, Glycogen synthase; SFA, saturated fatty acids; USFA, unsaturated fatty acids. See the text for detailed explanation and other abbreviations. Created in BioRender. Inspired by Wu & Ballantyne (2017).

1.2.3 Mechanism of lipotoxicity at the cellular level

Lipotoxicity is a metabolic condition that occurs primarily in insulin sensitive organs, such as the kidneys, liver, heart, and skeletal muscle, but also in the pancreas (Lipke et al., 2022). Interestingly, the most deleterious lipotoxic effects are provided by saturated FA, whereas unsaturated FA relieve cells from lipotoxic stress (Miller et al., 2005; Peng et al., 2011; Ricchi et al., 2009). Cardiac lipotoxicity can lead to several cellular mechanisms that can impair cardiac functioning (Wende et al., 2012). Below, I discuss oxidative stress, ER stress, and palmitoylation.

Oxidative stress is a term referring to an imbalance between oxidizing and reducing agents with a shift towards an oxidizing environment, due to an increased content of reactive oxygen species (ROS) (Betteridge, 2000). The family of nicotinamide adenine dinucleotide phosphate oxidases (NOX), is responsible for the transfer of electrons through biological membranes and plays an important role in the production of ROS (Bedard & Krause, 2007). ROS are also

produced during oxidative phosphorylation (Lambertucci et al., 2008). FA are known to be one of the factors inducing ROS production, and acute palmitate exposure increases superoxide production via these pathways (Lambertucci et al., 2008; Schönfeld & Wojtczak, 2008). Increased concentrations of palmitate have been reported to cause mitochondrial DNA damage and contribute to dysfunctional mitochondria, further compromising oxidative phosphorylation and initiating the apoptotic machinery (Rachek et al., 2007; Yuzefovych et al., 2013). Moreover, failure to detoxify ROS results in a free radical attack on proteins, lipids, carbohydrates, and nucleic acids, irreversibly altering their function or even destroying them completely (Lushchak, 2014). ROS also activates kinase pathways that are associated with cardiac hypertrophy and loss of function (Molkentin, 2004; Sawyer et al., 2002; Takimoto & Kass, 2007). ROS can impair calcium homeostasis, which can damage excitation-contraction coupling, and it is involved in the activation of NF-κB and the uncoupling of ATP production and oxygen consumption through the activation of uncoupling proteins (Boudina & Abel, 2006; Boudina et al., 2007; Fauconnier et al., 2007; Guzzardi & Iozzo, 2011; Hirotani et al., 2002).

ER stress is defined as a disturbance in ER homeostasis. The ER is involved in a wide range of biochemical and physiological processes, such as protein synthesis, post-translational modification and trafficking, but also regulating calcium homeostasis and lipid metabolism (Han & Kaufman, 2016). During ER stress, a collection of conserved, intracellular signalling pathways, called the unfolded protein response (UPR), are activated (Han & Kaufman, 2016). The UPR is an adaptive and protective mechanism aimed at restoring normal ER function. A prolonged activation of the UPR, however, can eventually lead to apoptosis, and accumulating evidence have linked lipotoxicity to ER stress and the UPR (Han & Kaufman, 2016).

In the ER, proteins fold into their native conformation. β-sheets are hydrophobic parts that usually form the core of proteins. But when proteins are unfolded, and these sheets are exposed to the ER, it activates the UPR. The UPR is controlled by three ER-resident membrane proteins, inositol-requiring enzyme-1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase R-like endoplasmic reticulum kinase (PERK) (Ron & Walter, 2007). In response to ER stress, the IRE1, ATF6 and PERK signalling pathways are activated to restore ER homeostasis through the activation of chaperone genes involved in protein translocation, folding, transportation and degradation (Lee et al., 2003).

Thus, the UPR is meant to resolve ER stress. But the increased FA uptake by cardiomyocytes during obesity and lipotoxic stress, has been shown to activate the IRE1 signalling pathway,

which can activate JNK, thereby inducing inflammation, insulin resistance and eventually cell death (Samuel & Shulman, 2012; Yamamoto et al., 2020). IRE1 and PERK, also phosphorylate eukaryotic translation initiation factor 2α (eIF2α), which antagonizes SREBP and thereby increase cholesterol and FA biosynthesis. In addition, phosphorylated eIF2α, leads to the activation of transcription factor ATF4, which, amongst others, activates the transcription factor DNA damage-inducible transcript 3 (CHOP). CHOP activates the transcription of genes involved in growth arrest and cell death (Ron & Walter, 2007). Furthermore, prolonged FA-induced ER stress impairs autophagy, the degradation and recycling machinery of the cell, which, if not resolved, leads to cell death (Park et al., 2015; Yorimitsu et al., 2006). Thus, saturated FA have shown to induce myocardial ER stress both *in vitro* and *in vivo*, which is associated with the activation of apoptotic signalling pathways, impairment of autophagy, and the inhibition of the sequestration of FA to TAG in lipid droplets, which are less harmful then DAG and ceramides (Bosma et al., 2014; Palomer et al., 2014; Park et al., 2015; Yang et al., 2019; Zou et al., 2017).

Palmitoylation is a post-translational modification process where palmitate-CoA is linked to the thiol group of certain cysteines (Martin et al., 2012). It is a reversable and dynamic process that is involved in several biological functions, and regulated by enzymes that continuously attach and detach palmitate moieties to protein cysteines (Schianchi et al., 2020). The binding of a palmitate moiety to a protein enhances its hydrophobicity and thereby its membrane affinity (Greaves & Chamberlain, 2007). The hydrophobic nature of the palmitoylated protein is crucial for directing these proteins to specific subcellular locations. The protein's location determines its involvement in the regulation of processes that are important for cellular functioning, such as protein trafficking between intracellular compartments, protein-protein interaction, and the stabilization of essential membrane proteins (Greaves & Chamberlain, 2007).

Although little is known about palmitoylation in the heart, studies in other cell types have shown that the palmitoylation of several proteins (including GLUT4 and CD36 themselves) are of crucial importance in the insulin signalling pathway and the subsequent translocation of GLUT4 and CD36 from endosomes to the membrane (Schianchi et al., 2020). However, since palmitoylation is substate-driven, lipid overload will lead to an excessive palmitoylation of proteins, and this can negatively regulate insulin signalling (Schianchi et al., 2020). For example, palmitoylation of PKCε, diminishes the gene expression of IR in skeletal muscles cells (Dasgupta et al., 2011), and hyper-palmitoylation of GLUT4 is observed in adipocytes of

obese mice (Ren et al., 2013). Hyper-palmitoylation of CD36 in the liver has been shown to negatively impact insulin sensitivity by increasing its translocation to the membrane, thereby enhancing FA uptake, while at the same time inhibiting the activation of AMPK, thereby reducing FA oxidation (Zhao et al., 2018).

1.3 The gut microbiota and its role in obesity and metabolic disorders

As shown in Figures 2 and 4, FA are not the only molecules that can bind to TLR. Lipopolysaccharide (LPS) are glycolipids and bacterial toxins that can also activate TLR and induce inflammation and insulin resistance. LPS is found on the outer membrane of gram-negative bacteria. The human gut is colonized by microorganisms such as bacteria, fungi, archaea, viruses, and protozoans and is collectively called the gut microbiome (GM) (Lynch & Pedersen, 2016). Most of these microbes are commensal or mutualistic and play an important role in the digestion of food, training of host immunity, regulating gut endocrine function and neurological signalling, eliminating toxins, modifying drug action and metabolism, and producing numerous compounds that influence the host (Lynch & Pedersen, 2016). The composition of the GM changes over time and is influenced by diet, physical activity, mode of birth, stool consistency, genetics, host immune system, infections, and use of antibiotics and other medication (Falony et al., 2016; Forslund et al., 2021; Maier et al., 2018; Pham & Lawley, 2014; Zhernakova et al., 2016). The GM is a complex microbial ecosystem in which microorganisms communicate, cross feed, recombine and coevolve (Layeghifard et al., 2017; Schroeder & Bäckhed, 2016). The dietary composition of the host and the presence of different microbes lead to the production of different microbial metabolites (Donia & Fischbach, 2015). These metabolites are important for microbial interaction and cross feeding, but can also affect the host physiology by binding to receptors or supplying the host with nutrients (Chen et al., 2019). In the last two decades the GM has received significant attention and it is now considered an important modulator of host physiology (Olofsson & Bäckhed, 2022).

1.3.1 The gut microbiome and metabolic disorders

Studies using germ free mice, antibiotic-induced microbiome-depleted mice, and faecal transplantations in mice and human, have all demonstrated the causal involvement of the GM in the regulation of host metabolism (Bäckhed et al., 2004; Carvalho et al., 2012; Ridaura et al., 2013; Vrieze et al., 2012; Zarrinpar et al., 2018). The GM affects the ability to digest and absorb nutrients, extract energy, and excrete by-products from ingested food. Through this it can affect

intestinal permeability, intestinal mobility, and the secretion of gastrointestinal hormones. Figure 5 gives an overview how the GM can affect host physiology, both locally as well as peripherally, through the production of several metabolites, such as short-chain fatty acids (SCFA), secondary bile product, trimethylamine N-oxide (TMAO), amino acid derived metabolites, and LPS (Cani et al., 2007; De Mello et al., 2017; den Besten et al., 2015; Fan & Pedersen, 2021; Gummesson et al., 2011; Koh et al., 2018; Parada Venegas et al., 2019; Wahlström et al., 2016; White & Newgard, 2019; Zhu et al., 2016).

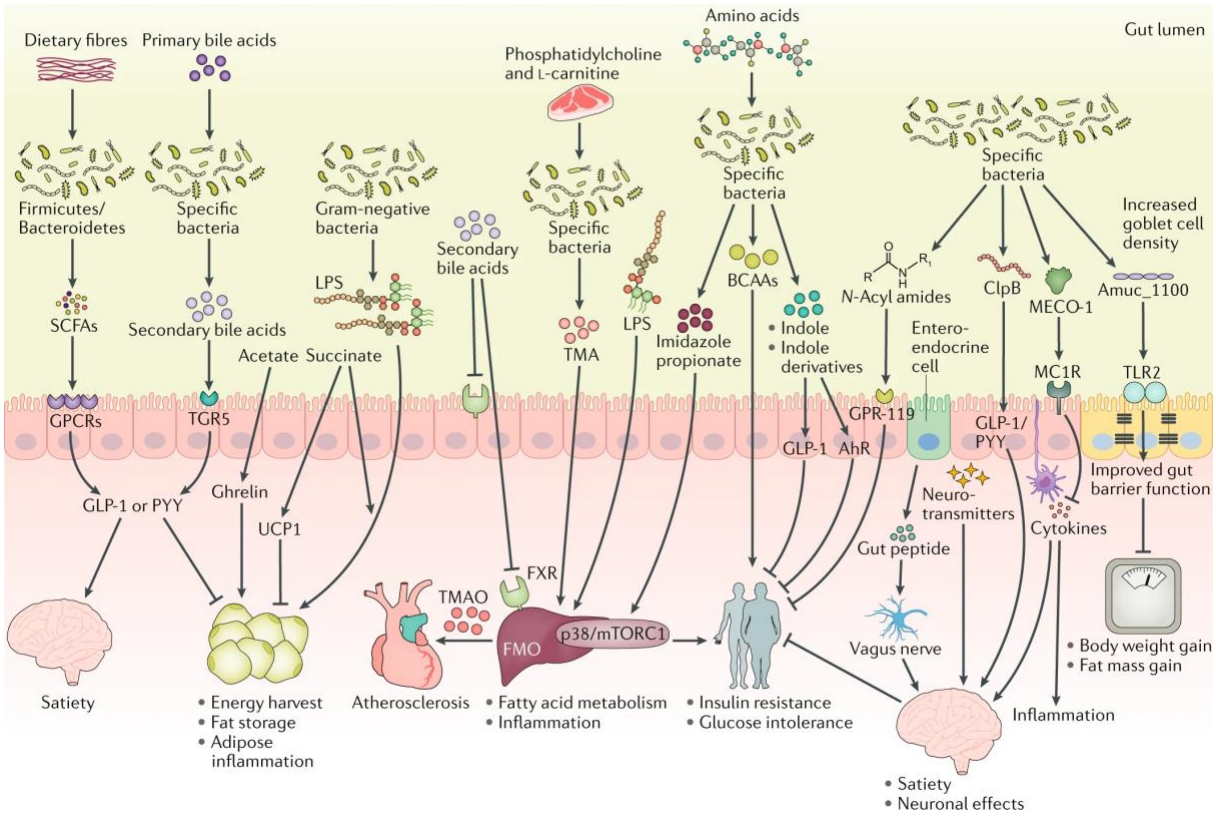

Figure 5: Microbial messengers regulate host metabolism. An overview of some of the intestinal microbial compounds affecting host energy homeostasis, body adiposity, inflammation, glucose regulation, insulin sensitivity and hormone secretion. BCAA, branched-chain amino acids; SCFA, Short-chain fatty acids; GLP-1, Glucagon-like peptide 1; TMA, trimethylamine; TMAO, trimethylamine N-oxide. Reprinted with permission from Fan and Pedersen (2021).

Although it is clear that the GM affects human physiology, it is less clear which composition, genera, species, or strains are health-promoting and which are pathogenic. Several studies have investigated the relationship between the faecal microbial composition and obesity in humans, but few consistent findings have been observed, due to the fact that most of the studies were underpowered (Sze & Schloss, 2016). Also, to study the relation between an unhealthy

microbial composition and metabolic disorders, one assumes to know what a healthy composition is. But this composition has yet to be defined (Fan & Pedersen, 2021). The relative distribution of the GM is unique to an individual, due to strain-level diversities, differences in microbial growth rates, structural variants within the microbial genes, and due to the influence of the host genetics and exposure to inter-individual variation in the environment, such as diet, physical activity, and medication (The Human Microbiome Project Consortium, 2012; Korem et al., 2015; Rothschild et al., 2018; Zeevi et al., 2019).

There is no "golden standard" of a human GM with the capacity to promote metabolic health of the host (Fan & Pedersen, 2021) But a comparative study between individuals from traditional living populations and modernized populations who consumed a western diet, showed a decline in microbial diversity and gene richness in the modernized populations (Clemente et al., 2015; Smits et al., 2017; Tyakht et al., 2013). This decline in diversity and gene richness has shown to correlate with an increased fat deposition, dyslipidemia, inflammation, and insulin resistance (Le Chatelier et al., 2013; Yang et al., 2015). But without clinical intervention studies aimed to restore the diversity of the GM in patients suffering from metabolic disorders, it is difficult to evaluate whether the decline in microbial gene richness is the cause of the disease, or the consequence (Cotillard et al., 2013). However, there are some common patterns observed regarding changes in the composition of the GM and functional features in different metabolic disorders (summarized in Figure 6).

1.3.2 Dietary interventions – effect of marine oils

One of the environmental factors that can affect the composition of the GM is the diet. The effect of diet on the GM, and in particular dietary fat, has been extensively studied in relation to metabolic health (Arumugam et al., 2011; David et al., 2014; Wu et al., 2011; Zhernakova et al., 2016). Wolters et al. (2019) performed a systematic review to study the quantity and quality of dietary fat intake on the human GM by analysing both intervention and observation studies. Surprisingly, based on the intervention studies, they found that dietary fat did not have a strong effect on the GM, or metabolic health outcomes, in humans. Their main findings were that diets high in total fat, saturated fatty acids, and monounsaturated fatty acids (MUFA) decreased total bacterial diversity and richness, while omega-3 polyunsaturated fatty acid (n-3 PUFA) did not appear to have any effect on richness and diversity. Also a later randomized trial studying the effect of n-3 PUFA did not find a change in alpha or beta diversity, or phyla composition upon EPA and DHA supplementation. Although they did observe an increase in the beneficial bacteria genera *Bifidobacterium, Roseburia* and *Lactobacillus* with n-3 PUFA intake (Watson

et al., 2017). Thus, to avoid any negative influences on bacterial diversity and richness in a high fat diet, saturated fatty acids should be replaced with n-3 PUFA.

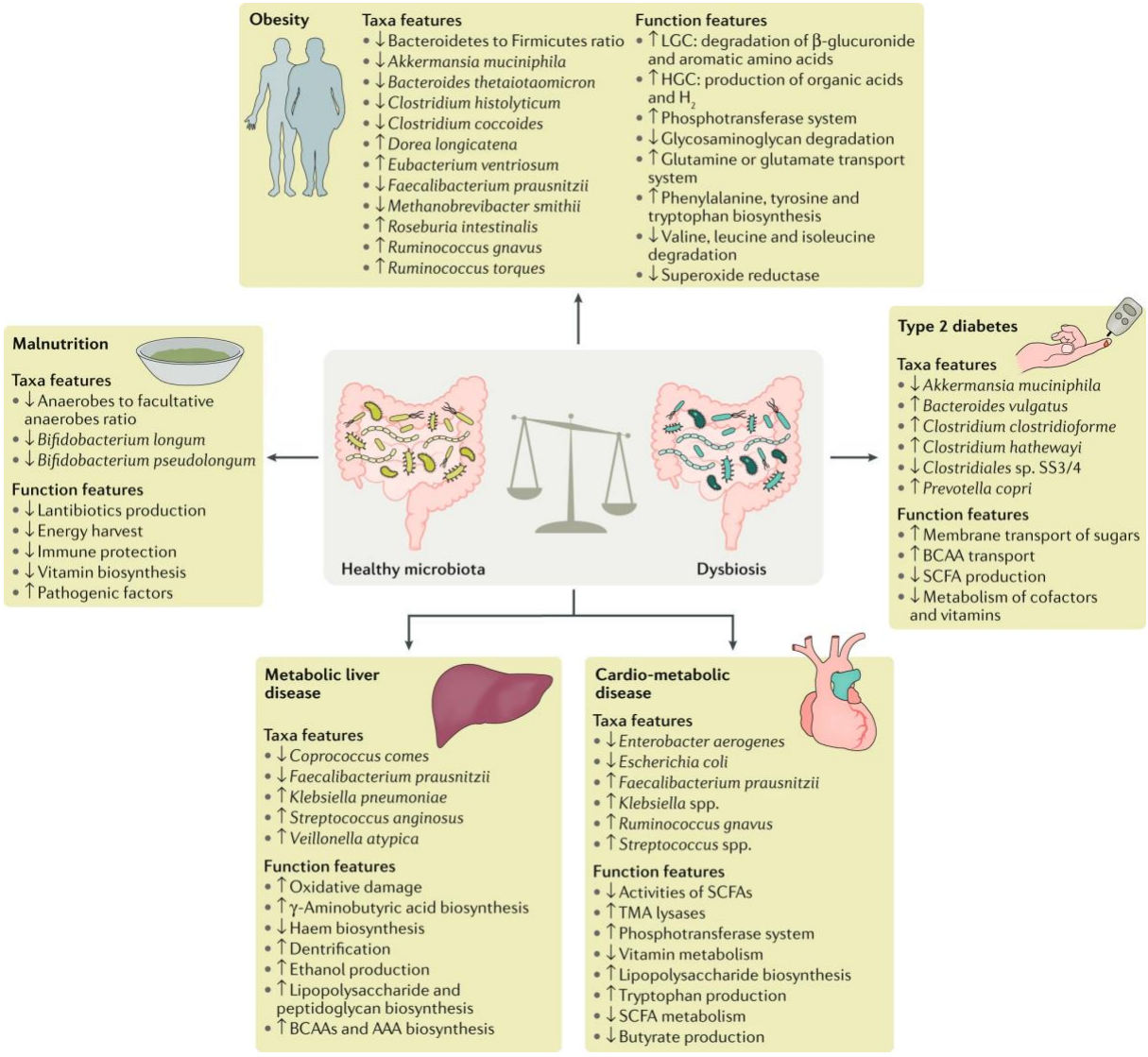

Figure 6. An overview of selected key gut microbial taxonomic and functional features related to metabolic diseases. AAA, aromatic amino acids; BCAA, branched-chain amino acids; HGC, high gene count; LGC, low gene count; SCFA, short-chain fatty acids; TMA, trimethylamine; ↓, lower abundance in metabolic diseases when compared with control; ↑, higher levels in metabolic diseases when compared with control. Reprinted with permission from Fan and Pedersen (2021).

1.4 Calanus oil – a novel marine oil

The n-3 PUFA that are present in seafood (EPA and DHA), have health-promoting properties in several human diseases and conditions. The American Heart Association, World Health Organisation and European Food Safety Authority therefore recommend a daily intake of about 250 mg/day EPA and DHA, or two servings of fish per week (EFSA, 2009; Rimm et al., 2018; WHO, 2003). The consumption of fish, and the use of fish oil as a nutraceutical by humans, as well as in aquaculture feed, has increased in the past decades (Tocher, 2015). This has put pressure on sustainable fisheries while there is still a gap between supply and demand for these marine lipids (FAO, 2022; Tocher, 2015). Thus, there is a need for a new and sustainable source of marine oils. Possible alternatives are the utilization of lower trophic level sources, such as the cultivation of marine microalgae, or the harvest of zooplankton like krill and calanoid species (Shepherd & Jackson, 2013; Tocher, 2015; Tocher et al., 2019).

Calanus finmarchicus is a marine crustacean belonging to the order Copepoda and it is one of the most prominent zooplankton species, in terms of biomass, in the North Atlantic (Falk-Petersen et al., 2009; Falk-Petersen et al., 1987; Prokopchuk & Sentyabov, 2006). This copepod is approximately 3-4 mm in length and has a one-year life cycle. In spring and summer it feeds on phytoplankton blooms and spawns in the upper water layer (Diel & Tande, 1992). By late summer and autumn, this new generation of *C. finmarchicus* has built up a substantial lipid reserve in its oil sac and migrates to depths of 500-2000 m to hibernate and overwinter. During winter, *C. finmarchicus* matures and produces gonads before resurfacing again in spring and early summer to spawn (Falk-Petersen et al., 2009). It is during the summer months (when *C. finmarchicus* builds up its lipid reserves and aggregates at the ocean's surface), that this animal is commercially harvested for its lipid content, and from which Calanus oil is produced (Pedersen et al., 2014b).

1.4.1 Calanus oil – a unique chemical composition

The lipid fractions of Calanus oil consist of more than 85% of wax ester, which are formed by the esterification of a FA to a fatty alcohol. Phospholipids, TAG, cholesterol and non-esterified FA constitute only minor fractions in the oil (Pedersen et al., 2014b). The composition of the lipid classes makes Calanus oil unique compared to traditional fish and cod liver oil, where the FA are generally bound to glycerol, forming TAG, or in krill oil, where a large part of FA are present as phospholipids. The composition of the different lipid classes changes throughout the year, depending on the copepod's life-cycle stage at the time of harvest, season, and location

(Falk-Petersen et al., 1987). The average composition of the most important FA and fatty alcohols with regard to human health, as well as the sum of the saturated and unsaturated FA and fatty alcohols in commercially available Calanus oil is given in Table 1. This table shows that the content of EPA and DHA in this oil is relatively low, while the content of stearidonic acid (SDA), which is another n-3 PUFA, and MUFA is relatively high.

Table 1: Most important fatty acids and fatty alcohols (mg/g lipid) in commercially available Calanus oil. Modified from Pedersen et al., (2014a)

Fatty acid	**Content (mg/g lipid)**
16:0 (Palmitic acid)	45.1
18:1n-9 (Oleic acid)	15.5
20:1n-9 (Gondoic acid)	24.0
22:1n-11 (Cetoleic acid	43.3
18:4n-3 (Stearidonic acid)	69.6
20:5n-3 (Eicosapentaenoic acid)	54.7
22:6n-3 (Docosahexaenoic acid)	39.4
Σ SFA	112.3
Σ MUFA	110.9
Σ n-3 PUFA	180.3
Σ Fatty acids	**412.3**
Fatty alcohol	
20:1n-9 (Eicosenol)	128.8
22:1n-11 (Docosenol)	188.1
Σ Fatty alcohol	**348.0**

1.4.2 Digestibility of Calanus oil wax esters

For Calanus oil to be an alternative source of marine lipids, the wax esters need to be digestible, and the FA and fatty alcohols need to be bioavailable. There have been concerns about the digestibility of wax esters in humans (Ling et al., 2009). However, feeding experiments with Calanus oil have shown the presence of wax ester-derived fatty alcohols in the faeces, confirming the hydrolyses of the wax esters. The increased incorporation of n-3 PUFA (including SDA) in erythrocyte membranes, liver and adipose tissue in mice, as well as the increased omega-3 index and the presence of fatty alcohols in the circulation in humans, confirms the absorption of the hydrolysed wax esters (Brezinova et al., 2020; Jansen et al., 2019; Pedersen et al., 2014a; Štěpán et al., 2021). The bioavailability of Calanus oil has been tested clinically in volunteers consuming 4 g/day (Cook et al., 2016), without any ill effects, although the recommended amount is 2 g/day (Tande et al., 2016). The short-term

bioavailability in humans was documented by measurements of the plasma concentration of EPA and DHA, while long-term bioavailability was confirmed by measurements of the omega-3 index and proved to be comparable to that of other marine oils (Burhop et al., 2022; Cook et al., 2016; Vosskötter et al., 2023; Wasserfurth et al., 2020a).

1.4.3 Health effects of Calanus oil

Experimental studies using mice fed a high-fat diet (HFD) reported reduced body weight gain and improved metabolic and inflammatory parameters when the diet was supplemented with 1-2% Calanus oil (Höper et al., 2013). A follow-up study showed that these effects could be attributed to the wax esters in the oil (Höper et al., 2014). Dietary supplementation with the oil has further shown to reduce atherosclerotic lesion formation, prevent angiotensin-II induced hypertension, and protect the heart from damage during ischemia-reperfusion (Eilertsen et al., 2011; Jansen et al., 2019; Salma et al., 2016).

Human studies have also reported beneficial health effects of Calanus oil. In healthy individuals 2 g/day Calanus oil over 12 weeks, combined with exercise, led to increased body fat loss compared to exercise alone (Wasserfurth et al., 2020b). In contrast, the same supplementation for six months in healthy, physically-fit normal to overweight men and women, did not show any effect on maximal oxygen uptake (Tauschek et al., 2022). In obese prediabetic individuals, however, 16 weeks with 2 g/day Calanus oil was found to improved glucose homeostasis and hepatic insulin resistance (Burhop et al., 2022). Likewise, exercise combined with 2.5 g/day Calanus oil for 16 weeks improved cardiorespiratory function, and functional strength of the lower body in elderly women, compared to exercise in combination with a placebo oil (Daďová et al., 2022; Štěpán et al., 2021).

1.4.4 Health effects of the different components of Calanus oil

Upon consumption, the wax esters are hydrolysed to their corresponding FA and fatty alcohols in the gastrointestinal tract (Hargrove et al., 2004). These wax esters consist of a cocktail of FA and fatty alcohols, as shown in Table 1, and many of the n-3 PUFA and MUFA present in the oil are known to have beneficial effects on human health.

EPA and DHA supplementation has been shown to reduce adipose tissue mass and systemic inflammation in obese non-diabetic patients and are associated with lower plasma levels of inflammatory biomarkers in type 2 diabetes patients (Itariu et al., 2012; Natto et al., 2019). They are also reported to reduce hypertriglyceridemia and the risk of coronary heart disease and cardiovascular disease when given at dose of 4 g/day (Hu et al., 2019; Rimm et al., 2018;

Skulas-Ray et al., 2019). The mechanisms behind these observed health effects are not fully understood, but n-3 PUFA are known to have anti-inflammatory properties. They can change the phospholipid composition of the cell membrane by competing with arachidonic acid as a metabolic substrate. This can change the production of prostaglandins, thromboxanes, and leukotrienes from a pro-inflammatory to a more anti-inflammatory state, and increase the production of resolvins, protectins and maresins. In addition, these n-3 PUFA can change the activation of different transcription factors in an anti-inflammatory direction (Calder, 2015).

SDA is another, lesser studied, n-3 PUFA. SDA is rapidly converted to EPA, and it is believed to have anti-inflammatory properties by binding to the G-protein-coupled receptor (GPR) 120 in macrophages, adipocytes, and the gut (Christiansen et al., 2015; James et al., 2003; Kotarsky et al., 2003).

MUFA have also been associated with a reduced risk to develop metabolic diseases (Gillingham et al., 2011; Pérez-Jiménez et al., 2002). The interests in the health effects of MUFA are based on the observation of a reduced risk for cardiovascular diseases in the adult population in regions bordering the Mediterranean Sea, where the consumption of olive oil (rich in oleic acid) is high (Delgado-Lista et al., 2016). Although it is not fully understood how MUFA exert their health effects, they have shown to reduce adipocyte size. In addition MUFA are known to be PPAR-γ ligands and supplementation with these FA was shown to increase the gene expression of PPAR-γ and its target genes in the liver and adipose tissue (Grygiel-Górniak, 2014; Yang et al., 2016; Yang et al., 2017; Yang et al., 2013).

Fatty alcohols found in Calanus oil (eicosenol and docosenol) have not previously been studied in relation to obesity and metabolic disorders. Policosanols, which are very long-chain fatty alcohols with a backbone longer than 22 carbons, however, have been studied. It has been reported that policosanols have anti-inflammatory properties, improve blood lipid profile and insulin sensitivity, and reduce body fat, while increasing thermogenesis in brown adipose tissue through the activation of GPR120 in adipocytes (Cho et al., 2018; Fernández-Arche et al., 2009; Hsu et al., 2015; Kim et al., 2017; Kim et al., 2018; Montserrat-de la Paz et al., 2014; Sharma et al., 2019).

2 Aims of the thesis

Calanus oil, derived from the marine crustacean *Calanus finmarchicus*, has a unique chemical composition in the sense that approximately 85% of the oil consists of wax esters, i.e. a fatty acid esterified to a long-chain monounsaturated fatty alcohol. The fatty acid component of the wax esters consists mainly of a cocktail of n-3 PUFA and MUFA, which have been reported to have beneficial health effects, whereas potential health effects of the specific fatty alcohols found in Calanus oil are less studied. The overall aim of this thesis was to get a better understanding of how Calanus oil and its constituents could possibly be used as a therapeutic approach to target obesity-induced metabolic distortions.

Paper I

The aim of paper I was to initially review previously published results regarding the impact of n-3 PUFA, MUFA, and policosanols on obesity-induced (low-grade) inflammation.

Paper II

Low-grade inflammation and obesity have been associated with changes in the composition of the GM. As previously shown, dietary supplementation with Calanus oil reduces diet-induced obesity in mice. The aim of paper II was therefore to find out if intake of Calanus oil (and reduction in body fat) was able to prevent obesity-induced changes in the GM.

Paper III

The wax esters present in Calanus oil are digested and hydrolysed to FA and fatty alcohols in the gastrointestinal tract before they are absorbed and enter the circulation. The aim of paper III was to develop a method to hydrolyse the wax esters and subsequently separate the FA from the fatty alcohols. This will allow to study the individual effects of these lipid fractions *in vitro*, and thereby to get a better understanding of the health effect of Calanus oil wax esters at the cellular level.

Paper IV (manuscript)

Calanus oil has previously been shown to reduce diet-induced obesity and to be cardioprotective in mice. The aim of paper IV was to find out if Calanus oil wax ester-derived FA could prevent palmitate-induced lipotoxicity in an *in vitro* model of H9c2 cardiomyoblasts, focusing primarily on cell viability and fatty acid oxidation.

3 Methodological considerations

3.1 Animals and dietary regime

In paper II we examined the effect of obesity on the GM, and whether anti-obesogenic treatments (i.e. dietary supplementation with Calanus oil or administration of the GLP-1 receptor agonist, exenatide) could prevent the anticipated obesity-induced changes in the composition of the GM. We used C57Bl/6J mice (Charles River, Sulzfeld, Germany) as our experimental model, since this strain is particularly susceptible to diet-induced obesity due to a mutation in the nicotinamide nucleotide transhydrogenase gene (Collins et al., 2004; Nicholson et al., 2010; Surwit et al., 1988). To induce obesity, mice received a HFD containing 45% energy from fat (HFD, no, 58V8, Test Diet; IPS limited, Richmond, Indiana, USA) over a 12-week feeding period. In the following 8 weeks the mice received a HFD in which 2% lard had been replaced with 2% Calanus oil, or a HFD combined with subcutaneous administration of exenatide (10 μg/kg/d via mini-osmotic pumps). The obese and lean control mice received a HFD and an ordinary chow diet, respectively, throughout the whole 20-week feeding period. The experiments were approved by the local authority of the National Animal Research Authority in Norway.

We realize that the duration of this experiment could have been longer, because the degree of obesity/body weight gain in response to the HFD was markedly lower than in previous, and more prolonged, studies reported by our group (Höper et al., 2014; Höper et al., 2013). Also, longer exposure to the treatments (Calanus oil and exenatide) could probably have revealed more clear changes of the GM. Finally, we examined only samples from the colon, and it cannot be excluded that samples from other parts of the intestine (e.g. the cecum) could have given other results.

3.2 Next generation sequencing

Our collaborators at Institute of Animal Physiology and Genetics of the Czech Academy of Sciences performed the bacterial gene sequencing experiments. They chose 16S rRNA sequencing over Shotgun Metagenomic Sequencing since this was their field of expertise. After 16S rRNA amplification and next generation sequencing, the sequences were clustered and identified by performing closed-reference operational taxonomic unit (OTU) picking against the Greengene reference OTU. Although a more common method is to use an open-reference OTU, the bioinformaticians at UiT The Arctic University of Norway, who assisted us with the analysis, recommended the closed-reference OTU because this would allow us to use the same

dataset to analyse both the taxonomic microbial profile, as well as the functional microbial profile. Since next generation sequencing and bioinformatics were not our fields of expertise we relied on the experience and recommendations of our collaborators and the methods they were most familiar with. However, it cannot be excluded that we would have acquired slightly different results if other methods had been used.

3.3 Separation of Calanus oil wax ester-derived fatty acids and fatty alcohols

The separation of the Calanus oil wax ester-derived fatty acids (FAC) and fatty alcohols was done using solid phase extraction (SPE) following the protocol described in Figure 7.

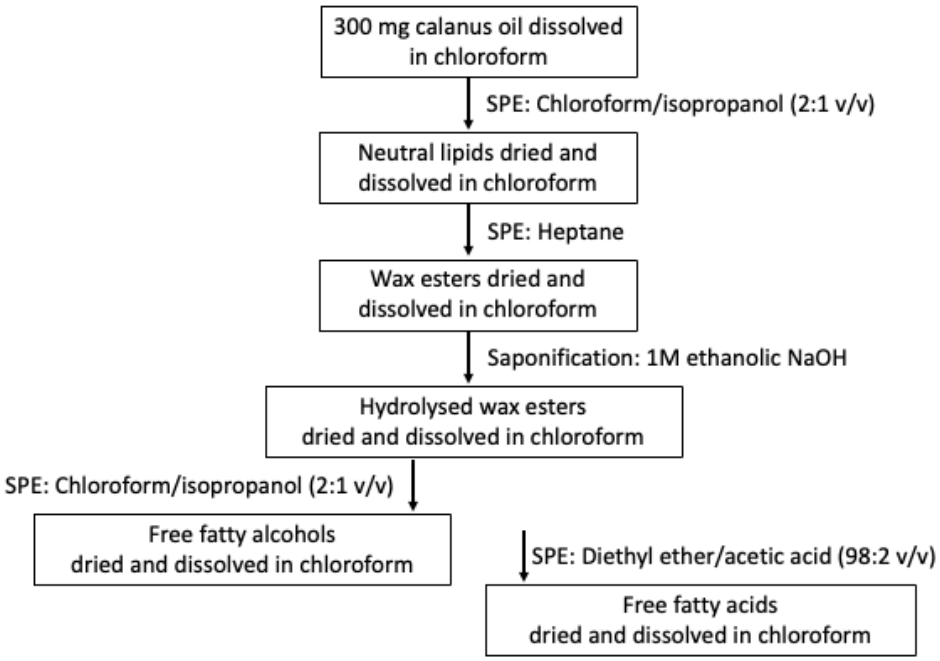

Figure 7: Isolation of fatty acids and fatty alcohols from Calanus oil wax esters by use of solid-phase extraction. Modified from Schots et al., (2023).

The separation of the wax esters was done following Vang et al. (2013), but with slight modification to optimize the isolation. For each SPE step, in which different lipids were eluted, the optimal elution volumes were qualitatively assessed using thin layer chromatography (TLC). Figure 8 is an example of the optimalization from the first SPE step, where the neutral lipids were eluted from the oil with chloroform/isopropanol (2:1 v/v). As can be seen, the majority of the neutral lipids were eluted with 10 mL chloroform/isopropanol (2:1 v/v). We used, however, 30 mL to optimize the yield.

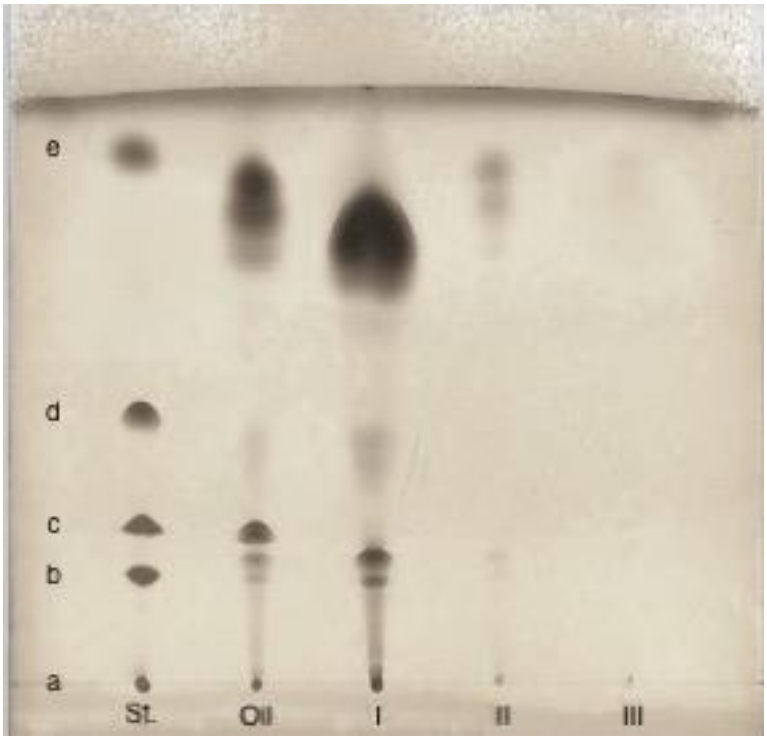

Figure 8: TLC of neutral lipids separated by SPE from 300 mg Calanus oil. St.: fatty acid standard 18-5 A (Nu-Chek Prep, INC. USA), containing lecithin (a); cholesterol (b); oleic acid (c); TAG (d); and cholesteryl oleateI). Oil: oil from *C. finmarchicus*. Lane I: Neutral lipids (NL) eluted with 10 mL chloroform/isopropanol (2:1 v/v), Lane II: NL eluted with an additional 10 mL (20 mL in total), Lane III: NL eluted with a final additional 10 mL (30 mL in total).

An alternative method to separate the FA from the fatty alcohols is also through the formation of a soap with aqueous NaOH but followed by the addition of heptane. The FA bind to sodium and solidify, while the fatty alcohols stay in solution and migrate to the upper heptane phase, allowing the collection of the isolated fatty alcohol fraction by removal of this heptane layer. The addition of an acid to the soap will dissociate the sodium from the FA. A subsequent second addition of heptane will result in a new heptane phase with dissolved FA. We have tried this method using different protocols with both NaOH and KOH, but the separation of the FA and fatty alcohols was not as clear as by use of SPE.

3.4 H9c2 cardiomyoblasts

H9c2 cardiomyoblasts were originally derived from embryonic heart tissue from a female rat in 1976 (Kimes & Brandt, 1976). It is a relatively easy cell type to work with, having a high proliferation capacity, and our research group has long experience working with this cell line. The cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL

streptomycin. The cells were incubated in 5% CO_2 at 37°C. We used cells from consecutive passages 16 to 25, since cell line aging can cause variability in the results (Witek et al., 2016). Previous, unpublished, results by our research group have shown a cardioprotective effect of hydrolysed Calanus oil wax esters on palmitate-induced lipotoxicity in H9c2 cells. With our successful method to separate the FA from the fatty alcohols in the hydrolysed wax esters (paper III), we now wanted to test the protective effect of FAC, as well as specific n-3 PUFA (EPA, DHA, and SDA), and specific MUFA (cetoleic acid, gondoic acid, and oleic acid) present in the FAC.

3.5 Induction of lipotoxicity and assessment of cell viability

Lipotoxicity was induced by incubating the cells with palmitate. We initially assessed cell viability of H9c2 cells after a 20-hour incubation period with 100 μM and 200 μM palmitate. We chose to continue our experiments with a concentration of 100 μM palmitate since this concentration reduced cell viability to approximate 50% of control cells. Palmitate-exposed cells were co-incubated with different concentrations of FAC (ranging from 1.25-20 μM) or with different concentration of the individual FA: EPA, DHA, SDA, cetoleic acid, gondoic acid and oleic acid (ranging from 0.5-20 μM). FAC and the individual FA (including palmitate) were dissolved in ethanol, and cells incubated in the presence of ethanol (vehicle) served as controls, with a final 2% ethanol exposure to all cells. The cells were plated 24 hours prior to palmitate exposure in a 96-well plate with 5000 cells/well. This cell density was found (in pilot experiments using an MTS assay kit) to be optimal for assessment of cell viability. Using a 96-well plate allowed for several treatments, as well as using 2 to 3 replicates per treatment.

In pilot experiments, cell viability was initially assessed using the following methods: xCELLigence real-time cell analysis, MTS assay kit, and IncuCyte® Cell Proliferation Assay. The xCELLigence instrument measures the electrical conduction through gold microelectrodes that are fused to the surface at the bottom of microtiter plate wells. An increase in cell number will decrease the electron flow through the plate and thereby reduce the current. The cell adherence is given as a Cell Index (CI), which is the difference between the electrical impedance at the time point of interest and the impedance in the absence of cells, divided by a nominal impedance value. xCELLigence is a useful and rapid tool to measure cell proliferation and cell death in real-time. The main reason for not choosing this method, however, is the high cost. The MTS assay kit is an inexpensive and easy colorimetric way to assess cell viability. The limitation of this method is that you only get one timepoint value, which therefore does not

allow normalization to time-point 0. This is an important limitation when there are differences in cell densities between different wells, which is something we have often experienced and detected using live-cell imaging (as described next). Finally, the IncuCyte® Cell Proliferation Assay is a live-cell imaging system that has a user-friendly in-built tool to quantify cell viability based on confluence. The cells are continuously kept in an incubator with optimal temperature and CO_2 conditions. It has also proven equally effective as the xCELLigence biosensor technology (Single et al., 2015). The confluence of each well was determined, and the change in confluence over time was given as a Confluence Index (CI) (i.e. relative to time 0). Since changes in assessment of confluence is a less established method for cell viability, we combined the real-time IncuCyte® Cell Proliferation Assay with the MTS Assay kit. We found a significant correlation between the absorbance measured with the MTS and the non-normalized percentage confluence at 20-hours assessed with the IncuCyte®. An example of this significant linear regression from one experiment is shown in Figure 9.

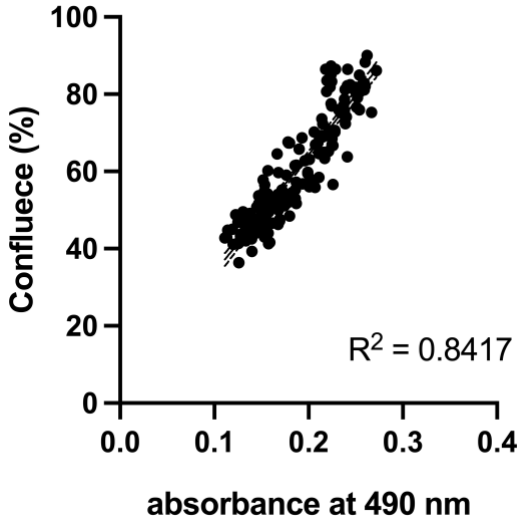

Figure 9: Linear regression between cell viability determined as confluence (using an IncuCyte® Cell Proliferation Assay) and absorbance at 490 nm (using an MTS assay kit). Each point represents a well from two 96 well plates. The correlation was significant with $p < 0.05$.

3.6 Assessment of fatty acid uptake and oxidation rates

The FA uptake and oxidation rates of H9c2 cell were assessed in 6-well plates during the first six hours of palmitate-exposure (i.e. before palmitate-induced cell death was observed). 2 μCi/mL ^{3}H-labelled palmitate was added as a tracer to the incubation medium and aliquots of 0.5 ml medium were taken at the start of treatment and every second hour over the 6-hour incubation period. The FA oxidation rate was based on the accumulative production of ^{3}H-labelled H_2O in the incubation medium. The ^{3}H-labelled H_2O in the samples was separated

from ^{3}H-labelled palmitate by vacuum sublimation. The FA uptake rate was assessed from the decline of ^{3}H-labelled palmitate in the incubation medium. The radioactivity from the samples was determined using liquid scintillation. The control cells in these experiments were incubated with a low dose of palmitate (1 μM), to be able to assess control values of FA uptake and oxidation. Values were normalized to the protein levels in the wells at the end of the 6-hour incubation period. The main benefit of taking multiple samples, as compared to only taking a sample at the start and the end of the incubation period (Haffar et al., 2015a), is that we can evaluate the time-course and potential changes in the respective rates over time, thereby obtaining steady-state measurement.

3.7 Assessment of gene expression

Quantitative real-time reverse transcription PCR was used to assess gene expression in papers II and IV. In paper II the mRNA expression of genes related to inflammation, cell wall permeability, and fat metabolism were evaluated in the colon and omental adipose tissue collected from mice. In paper IV we evaluated the mRNA expression of genes related to ER stress, inflammation, and lipid metabolism in H9c2 cells. The frozen colon samples in paper II, and the cells collected in paper IV were immersed in RNAlater, while the frozen adipose tissue samples in paper II were immersed in Allprotect Tissue Reagent and thawed overnight. RNA was extracted according to a RNeasy kit and the concentrations were measured spectrophotometrically using a Nanodrop 2000. The expressions of the target genes were normalized to the mean of the two housekeeping genes HPRT and SDHA.

3.8 Statistics

The data in paper II and IV are presented as the mean with standard error of the mean. In paper II a non-corrected 2-sided student t-test between the HFD group and the three other groups was performed to test for significant differences. Mean differences were considered significant with $p < 0.05$. The number of mice per group in paper II was relatively small ($n = 5$), which increases the risk of type II (false negative) errors. This was due to limited availability of samples, since this study was run in parallel to another study using the same animals (Jansen et al., 2019). In paper IV between group differences were analysed with a repeated measure one-way ANOVA, followed by a Dunnett's multiple comparison test. Data was significant with $p < 0.05$. Cells from one passage were considered as $n = 1$. All statistical analyses were done using GraphPad Prism 9.

4 Summary of results

Paper I: Possible Health Effects of a Wax Ester Rich Marine oil

Pauke Carlijn Schots, Alice Marie Pedersen, Karl-Erik Eilertsen, Ragnar Ludvig Olsen and Terje Steinar Larsen. *Frontiers in Pharmacology* 2020; 11 (961); doi.org/10.3389/fphar.2020.00961

This review was written to discuss the potential of Calanus oil as a metabolic therapy to treat or prevent obesity-induced low-grade inflammation and the consequent metabolic disturbances. Thus, the paper summarizes documented health effects, and mechanisms, of the different n-3 PUFA and MUFA present in the wax esters, as well as the less studied policosanols.

Main findings:

EPA and DHA have been reported to reduce adipose tissue mass and systemic inflammation. They can be used as a monotherapy to reduce hypertriglyceridemia, and supplementation with EPA and DHA can reduce the risk for coronary heart disease and cardiovascular disease. EPA and DHA replace arachidonic acid in the phospholipid bilayer of cell membranes and thereby alter the production of prostaglandins, thromobxanes, leukotrienes, resolvins, protectins and maresins. In addition, they can reduce the activation of pro-inflammatory transcription factors and increase the activation of anti-inflammatory transcription factors.

SDA can exert positive health effects through its relatively efficient conversion to EPA. SDA is also a potent ligand for GPR120, which is expressed in the colon, adipocytes, and macrophages. GPR120 can, amongst other things, inhibit the activation of NF-κB and thereby reduce the transcription of pro-inflammatory cytokines and adipokines, and it can activate the browning of white adipose tissue and thereby increase thermogenesis.

MUFA are potent ligands to PPAR-γ and can decrease the formation of atherosclerotic lesions, reduce cholesterol efflux, and alter the expression of genes related to inflammation, lipid metabolism and energy expenditure. In addition, cetoleic acid can improve the efficiency of the conversion of alpha-linolenic acid to EPA and DHA.

Policosanols can reduce body fat, improve lipid and glucose metabolism, and have anti-inflammatory properties. Some policosanols have shown to be able to activate GPR120 in adipose tissue and thereby induce browning and increase thermogenesis.

Paper II: Obesity-induced alterations in the gut microbiome in female mice fed a high-fat diet are antagonized by dietary supplementation with a novel, wax ester-rich, marine oil

Pauke C. Schots*, Kirsten M. Jansen*, Jakub Mrazek, Alice M. Pedersen, Ragnar L. Olsen and Terje S. Larsen. *Nutrition Research* 2020; 83 (94); *authors share first authorship
https://doi.org/10.1016/j.nutres.2020.09.002

The microbial composition of obese individuals is altered towards a more pro-inflammatory state, compared to lean individuals. Diet is one of the driving forces to change the GM. To study the effect of obesity on the GM, faecal samples from the colon were collected from HFD-induced obese mice, as well as from lean control mice receiving a normal chow diet. The main objective of this study was to find out if dietary supplementation with 2% Calanus oil or treatment with exenatide (both showing anti-obesogenic effects in mice), could revert the obesity-induced changes in the GM.

Main findings:

We found no significant differences in the microbial composition at the phylum level between the various dietary regimens. However, high-fat feeding did result in a change of the GM composition in an unhealthy direction, by increasing the abundance of the pro-inflammatory genera *Lactococcus* and *leuconostoc* and depletion of the health-promoting *Allobaculum* and *Oscillospira*.

Supplementing the high-fat diet with 2% Calanus oil did not significantly change the GM, compared to HFD-fed mice. There was, however, an apparent overrepresentation of the health promoting genus *Lactobacillus* and *Streptococcus*, the latter genus containing pro-inflammatory species as well as species considered probiotics. We also observed an apparent underrepresentation of the pathogenic considered genus *Bilophila.*

Administration of exenatide to HFD-fed mice was able to partly restore the microbial composition towards that of normal chow-fed mice. Exenatide decreased the HFD-induced increase in *Lactococcus,* and the abundance of *Streptococcus*.

Paper III: A simple method to isolate fatty acids and fatty alcohols from wax esters in a wax-ester rich marine oil

Pauke Carlijn Schots, Guro Kristine Edvinsen and Ragnar Ludvig Olsen. *Plos One* 2023; 18 (5);
https://doi.org/10.1371/journal.pone.0285751

Dietary supplementation with crude Calanus oil, and wax esters extracted from the oil, have shown to reduce diet-induced obesity and obesity-related metabolic disorders in mice. Wax esters are hydrolysed to FA and fatty alcohols in the gastrointestinal tract before they are absorbed and enter the circulation. To study the biological effects of the FA and the fatty alcohols, we realized that it was necessary to develop a method to hydrolyse the wax ester and separate the resulting FA and fatty alcohols.

Main findings:

SPE is a suitable semi-preparative method to isolate the wax esters from the other lipid classes present in Calanus oil and to separate the FA and fatty alcohols after hydrolysis. SPE is a simple method that does not require the methylation of the FA or the oxidation of the fatty alcohols to separate the two fractions and identify the composition.

From an average of 322 mg Calanus oil we obtained 75 mg fatty alcohols and 63 mg FA. The FA fraction was almost completely devoid of fatty alcohols, and in the fatty alcohol fraction less than 8% of the lipids were identified as FA.

Paper IV Calanus oil wax ester-derived fatty acids protect H9c2 cardiomyoblasts from palmitate-induced lipotoxicity

Pauke C. Schots, Wei Li, Andrea Stoltenberg, Trine Lund, Terje S. Larsen and Ellen Aasum (*manuscript*)

This study followed up on previous results from our lab which reported that Calanus oil supplementation has a cardioprotective effect in hearts from diet-induced obese mice. In addition, unpublished results showed that hydrolysed Calanus oil wax esters have a cardioprotective effect on palmitate-induced lipotoxicity in H9c2 cardiomyoblasts. In our study H9c2 cells were exposed to palmitate alone, or in combination with FAC (obtained through the method described in paper III), or EPA, DHA, SDA cetoleic acid, gondoic acid, or oleic acid. Cell viability, FA uptake and oxidation rates, as well as the gene expression of genes related to ER stress, inflammation, and lipid metabolism were assessed.

Main findings:

Palmitate exposure resulted in a time-depended decrease in cell viability, resulting in about 50% cell death after 20 hours. Co-incubation with FAC significantly, and in a dose-dependent manner, improved cell viability.

The individual n-3 PUFA and MUFA showed protective action against palmitate-induced lipotoxicity in varying degree, suggesting a synergistic effect, rather than an accumulated effect of the different unsaturated FA in FAC.

Palmitate exposure increased the FA uptake and oxidation rates compared to control cells, which coincided with an unaltered expression of GLUT4 and CD36, but a significant upregulation of PDK4 and CPT1.

Co-incubation with FAC resulted in a significant 20% further increase in the FA oxidation rate, without changing the FA uptake rate. This result was accompanied by a further increase in the expression of PDK4 compared to palmitate treatment alone.

5 Discussion

The aim of this PhD project was to obtain a better understanding of how Calanus oil and/or its constituents can possibly be used to target obesity-induced metabolic distortions. We confirmed that Calanus oil has a chemical composition that is different from other marine oil preparations. This is relevant to the previously documented anti-obesogenic and anti-inflammatory actions of the oil, as well as the currently reported impact on the composition of the GM. An important part of this thesis was the development of a method to separate the FA and fatty alcohols from hydrolysed Calanus oil wax esters. We believe that the purified preparations of these components will be useful tools to understand the mechanisms behind the protective effect of Calanus oil, especially with respect to preventing obesity-induced lipotoxic stress in the heart and possibly also other organs.

5.1 Microbiome

The intestine is the first organ that becomes exposed to Calanus oil intake, and this organ has been shown to be a target for the treatment of obesity and metabolic disorders. After consumption, the wax esters in the oil are hydrolysed to FA and fatty alcohols (Hargrove et al., 2004). Due to their hydrophobic nature, however, wax esters are more difficult to emulsify than most other lipid classes. The wax esters may therefore exhibit a longer retention time in the gastrointestinal tract to facilitate hydrolysis and absorption, and undigested wax ester may reach the lower part of the intestine before hydrolysis and absorption occurs (Cowey & Sargent, 1977; Verschuren & Nugteren, 1989). The role of the GM in human health has received significant interest in the last two decades, and changes in the composition of the GM have been associated with obesity and metabolic disorders, such as insulin resistance and cardiometabolic diseases (Boulangé et al., 2016; Graham et al., 2015; Olofsson & Bäckhed, 2022; Tindall et al., 2018; Winer et al., 2016). Since dietary supplementation with Calanus oil has previously shown to reduce obesity and obesity-related metabolic disorders in mice (Höper et al., 2014; Höper et al., 2013), we had anticipated that Calanus oil would also antagonize obesity-induced changes of the GM.

Our results confirmed previous reports, in that a HFD changed the microbial composition in an unhealthy direction (Hamilton et al., 2015; Ravussin et al., 2012). However, a 2% supplementation of the HFD with Calanus oil did not lead to any significant changes in the microbial composition, although we did observe a tendency towards a healthier phenotype by an overrepresentation of *Lactobacillus* and an underrepresentation of *Bilophila* compared to

HFD-fed mice. Our results are somewhat in line with those by Caesar et al. (2015) who also found a decreased abundance of *Bilophila* in male mice fed a HFD where 100% of the lard was replaced by fish (Menhaden) oil, and those by Mujico et al. (2013) who found an increase in *Lactobacillus* in female mice fed a HFD supplemented with 3000 mg/kg/day EPA and DHA. They also found that a HFD supplemented with 1500 mg/kg/day oleic acid could restore the HFD-induced microbial dysbiosis.

The fact that we did not observe a significant difference in the GM between mice given a HFD versus a HFD supplemented with Calanus oil was not completely unexpected. First, changes in the microbial composition occur mainly with short-term extreme dietary changes, such as in the studies by Caesar et al. (2015) and Mujico et al. (2013), with high dosages of unsaturated FA. Mild dietary changes on the other hand, such as replacement of 2% lard with Calanus oil, usually only results in minor compositional changes (Kolodziejczyk et al., 2019; Korem et al., 2017). Second, the treatment period was relatively short (8 weeks) and did not result in a reduced body weight, despite a reduction in the adiposity index.

Although we did not find any significant difference in the microbial composition of mice fed a HFD versus a HFD supplemented with Calanus oil, the differences that we did observe might still be of biological relevance. There is no standard for what is a healthy and unhealthy microbial composition (Fan & Pedersen, 2021), and it is therefore difficult to know how big a compositional change needs to be to affect the host physiology. One of the reasons for this is that the function of a genus or species depends on, and can change in, the presence or absence of other microbes, due to cross-feeding, recombination and coevolution (Layeghifard et al., 2017). Also, within a genus some species can be pathogenic, while others are probiotic, such as the genus *Streptococcus* and the specie *S. thermophilus*. But since not all studies are able to analyse the microbial composition on species level, this difference can be overlooked. Furthermore, different genera or species might have the same function, for example the production of SCFA from dietary fibre (Deleu et al., 2021; Macfarlane & Macfarlane, 2003). There are many different species that can produce SCFA. Non-significant changes in the abundance of several of these individual species can thereby still cause a significant change in the production of SCFA and affect the host. Thus, non-significant changes in the GM might still be important, while significant changes do not necessarily lead to functional alterations.

While replacing saturated FA by unsaturated FA has been shown to beneficially affect host physiology through alterations in the microbial composition in mice (Caesar et al., 2015), a

systematic review on the quality and quantity of dietary fat in humans did not report any alterations in the GM and the metabolic health of the host (Wolters et al., 2019). However, comparing results from different studies, such as in metanalyses and reviews, needs to be done with care. Diet is, for example, not the only driver to change the GM, energy intake is also such a driver, and the energy intake can vary between studies due to differences in fat intake. Additionally, an increase in dietary fat is usually paralleled with a decrease in carbohydrates, making it difficult to conclude which macronutrient is inducing the potential change in composition. Furthermore, most human intervention studies are of short duration and are not able to do a complete taxonomic assessment of the microbiome in the samples due to technical limitations. Technical limitations, as well as different methods used in sample collection, storage and preparation, DNA extraction, library preparation, the use of different reference databases or software programs, and even different mouse strains, animal vendors and baseline values can lead to biased results, making it difficult to compare data from different studies (Costea et al., 2017; Jovel et al., 2016; Montgomery et al., 2013; Rasmussen et al., 2019; Wesolowska-Andersen et al., 2014).

Due to the limitations in the current study (short treatment period, no marked reduction in body weight and few animals included), future studies are needed to determine the effect of Calanus oil on the GM. A follow up intervention study could be considered, in which GM analyses are performed before and after, rather than only at the endpoint of the intervention. Additional parameters should be measured in the faeces and in blood samples, such as LPS, SCFA, TMAO and secondary bile acids. As shown in Figure 5, it is mainly through metabolites and molecules that the microbiome can affect host physiology, and changes in these parameters are therefore arguably more important than changes in the microbial composition itself.

5.2 Isolation of Calanus oil wax ester-derived fatty acids and fatty alcohols

Most cells lack the enzymatic machinery to hydrolyse wax esters themselves. In order to study the separate effects of the FA and fatty alcohols in different organs or cell types, a method had to be developed (paper III) to isolate, and subsequent hydrolyse, the Calanus oil wax esters and to separate the FA and fatty alcohol fractions from each other.

Because of the importance of *Calanus finmarchicus* in the North Atlantic marine food web, several analytical methods have been developed to identify and quantify the FA and fatty alcohol composition in the lipid sac of *C. finmarchicus*. These methods, however, are not developed with the intention to separate the hydrolysed wax ester fractions, and often require

transesterification, derivatization, separation, and purification steps, involving the formation of fatty acid methyl esters (FAME) (Budge & Iverson, 2003; Kattner & Fricke, 1986; Webster et al., 2006). Therefore, a new method to isolate the native non-esterified FA and fatty alcohols had to be developed. This new method, using SPE, avoids modification steps, such as the formation of FAME followed by a second hydrolyses of the FA and methyl ester, which could potentially alter the composition or potency of the FA and fatty alcohols. We observed, for example, some minor differences in the FA composition between the FA in the wax esters and in the separated FA fraction. This indicates that some oxidation of the unsaturated FA may have occurred during the hydrolysis or separation steps. In addition, the weight of the hydrolysed wax ester was markedly reduced compared to the initial weight of the intact wax ester. These changes in the lipid composition and weight, using only one hydrolyzation step, supports the reasoning that additional modification stapes (formation and subsequent second hydrolyzation of FAME) are not optimal. To our knowledge, the method we have developed is the first semi-preparative successful procedure to separate wax esters and its hydrolysed components, leaving the isolated FA and fatty alcohols unaltered.

5.3 Calanus oil – more than an EPA- and DHA-rich marine oil

The method developed in paper III opens for the possibility to perform pre-clinical (animal models) and clinical studies to examine the effects of Calanus oil wax ester-derived FA and fatty alcohols separately. Moreover, information about the FA composition of the oil will also allow assessment of which FA contribute to the health effects observed in Calanus oil supplementation studies.

Feeding experiments in mice have shown that Calanus oil wax esters have potent anti-obesogenic, anti-diabetic and anti-inflammatory properties, and that replacement of only 1% lard with wax esters in the diet was sufficient to reduce HFD-induced obesity and obesity-related metabolic disorders (Höper et al., 2014). Marine oils rich in EPA and DHA have previously been shown to reduce adipose tissue mass (Itariu et al., 2012) but, as mentioned in the introduction, Calanus oil is relatively low in EPA and DHA, 6% and 4%, respectively (Pedersen et al., 2014a). It is therefore unlikely that the anti-obesogenic effects obtained with dietary supplementation of 1-2% Calanus oil in HFD-fed mice, as reported by Höper et al., (2014; 2013), were solely due to the EPA and DHA content in the oil. Most likely also other lipids (unsaturated FA or fatty alcohols) contribute to the health promoting effect of the oil. In addition, Calanus oil was shown to be cardioprotective (Jansen et al., 2019), which might also

suggest that other unsaturated FA play a role in the health effect of the oil. The cardioprotective role of EPA and DHA is an ongoing debate with discrepancies between and within observational studies and clinical trials. The details, which are somewhat beyond the scope of this thesis but still relevant for the discussion of cardioprotective action of omega-3 FA, have been placed in box 1.

5.4 Cardioprotective effects of Calanus oil wax ester-derived fatty acids

In paper IV, we studied the cardioprotective effects of FAC and the three most abundant n-3 PUFA and three most abundant MUFA, using H9c2 cardiomyoblasts as experimental model. The cells were incubated with 100 μM palmitate to induce lipotoxic stress and co-incubated with different concentrations of FAC, or one of the six unsaturated FA. Treatment with palmitate led to a time-dependent increase in cell death (after the first six hours), compared to vehicle-treated control cells. This was associated with an increase in the expression of CHOP and ATF4, two markers of ER stress, as well as IL-6, a marker for inflammation (two processes underlying the initiation of cell death). Our findings were in line with previous studies reporting palmitate-induced cell death and the involvement of ER stress in H9c2 cells (Akoumi et al., 2017; Park et al., 2015; Yang et al., 2019; Zou et al., 2017). Co-incubation with FAC had a dose-dependent protective effect on palmitate-induced cell death from 2.5 μM, and co-incubation with 10 μM FAC reduced the expression of CHOP and IL-6. The reduced expression in CHOP was similar to the reduced expression found by Yang et al. (2019) in palmitate-exposed H9c2 cells treated with 4-phenyl butyric acid, an inhibitor of ER stress, confirming the role of ER stress on palmitate-induced cell death. Haffar et al. (2015b) observed a palmitate-induced increase in the expression of IL-6 in primary rat neonatal cardiomyocytes, which was reduced in a similar fashion to our study by co-incubation with oleate, one of the main unsaturated FA found in FAC. These findings suggest that FAC is, partly, cardioprotective in palmitate exposed H9c2 cells by reducing ER stress and the activation of the immune system.

The FA uptake and oxidation rates were markedly increased following addition of palmitate to the incubation media of the cells, and this was associated with an increase in the expression of PDK4 and CPT1. Co-incubation with FAC led to a 20% further increase in the FA oxidation rate, which was associated with a further increase in the expression of PDK4. An increase in FA oxidation was also observed in palmitate-exposed primary rat neonatal cardiomyocytes co-incubated with oleate (Haffar et al., 2015a). Oleate is not the only unsaturated FA that has shown to have cardioprotective effects on palmitate-induced lipotoxicity. EPA and DHA have

also shown to be cardioprotective and they have been reported to reduce the palmitate-induced gene expression of DGAT1 and SPTLC1 (genes encoding for key enzymes in the formation of DAG and ceramides), and to restore the palmitate-induced decreased expression of CPT1 (Cetrullo et al., 2020). Depending on the energy needs of the cell, acyl-CoA can enter one of three competing metabolic processes; mitochondrial β-oxidation for ATP production, incorporation into glycerolipids (DAG or TAG), or formation of ceramides (which are precursors of sphingolipids) (Holland et al., 2007). An increased FA oxidation shunts the acyl-CoA into the first process, thereby potentially reducing the intra-cellular lipid accumulation via the other two processes, and thereby lipotoxic stress. We show in our study that FAC improves cell viability in palmitate exposed H9c2 cells. Based on our results, together with those of the above-mentioned studies, it can be suggested that FAC protects H9c2 cells from palmitate-induced lipotoxicity (reduces ER stress and activation of the immune system) by increasing the FA oxidation and thereby reducing the formation of DAG and ceramides. Future experiments are required to confirm this hypothesis.

Since FAC is a cocktail of different FA, where the content of the individual unsaturated FA ranges from 5-15%, we co-incubated palmitate exposed H9c2 cells with different concentrations of the six most abundant unsaturated FA for assessment of their impact on cell viability. The relative content of these FA in 10 μM FAC (which provides near full protection of palmitate-exposed cells) ranges between 0.5-1.5 μM, and so we tested the cardioprotective effect of the individual FA at low dosages (0.5-20 μM). All unsaturated FA had a dose-dependent cardioprotective effect on palmitate-induced cell death, and EPA was the most potent, followed by DHA and oleate. The viability of palmitate-exposed cells co-incubated with 20 μM of these FA was not different from that of control cells, but none of unsaturated FA had a beneficial effect on cell viability at the very low concentrations provided by 10 μM FAC. On the contrary, co-incubation at these low concentrations with gondoic acid, cetoleic acid and SDA had a negative effect on cell viability. Only at 10 μM were gondoic and cetoleic acid cardioprotective, while SDA was only protective at 20 μM.

In light of the studies with Calanus oil and its reported cardioprotective, anti-obesogenic and anti-diabetic effects, it needs to be highlighted once more that Calanus oil is different from other marine oils. The unique lipid composition of the oil constitutes a complex supplement that has the potential to improve metabolism via various mechanisms. Although EPA and DHA might play an important role regarding the oil's beneficial effects, the contribution of SDA and

the MUFA, oleic acid, gondoic acid and cetoleic acid should not be underestimated. These FA may have beneficial health effects thought their interaction with GPR120 or the different PPAR receptor proteins (Christiansen et al., 2015; Grygiel-Górniak, 2014; Kotarsky et al., 2003; Yang et al., 2016; Yang et al., 2017). Also, the rapid conversion of SDA to EPA, as well as cetoleic acid´s effect to increase the efficiency by which alpha-linolenic acid (and perhaps also SDA) is converted to EPA and DHA should be taken into account (James et al., 2003; Østbye et al., 2019; Østbye et al., 2023). Finally, our finding that none of the individual unsaturated FA were cardioprotective at the low concentrations provided by 10 μM FAC, in contrast to 10 μM FAC itself, suggests a synergistic effect of the different unsaturated FA in FAC, rather than an accumulated individual effect of the n-3 PUFA and MUFA. However, how these results can be extrapolated to humans is not yet clear, also because data regarding the plasma concentrations of the different n-3 PUFA and MUFA following the recommended intake of Calanus oil (2 g/day) are lacking. Although the study by Cook et al. (2016) did show that a bolus intake of 4 gram Calanus oil resulted in a (peak) plasma concentration of EPA and DHA of approximately 25 and 50 μg/mL (80 and 150 μM) respectively – which are well above the cardioprotective concentrations observed in our *in vitro* study.

5.5 Calanus oil wax ester-derived fatty alcohols

Since FA form only half of the wax ester molecule, it can be thought that fatty alcohols also contribute to the health effects of the Calanus oil wax esters. Although the health effects of the long-chain monounsaturated fatty alcohols found in Calanus oil (eicosenol and docosenol) have not yet been investigated in relation to metabolic diseases, policosanols have. The positive health effects of policosanols are debated (Marinangeli et al., 2010). However, recent *in vitro* and animal studies (as well as clinical studies using policosanols from different natural resources) have reported positive health effects with regard to adiposity, insulin resistance, lipid metabolism and inflammation (Fernández-Arche et al., 2009; Gong et al., 2018; Hsu et al., 2015; Montserrat-de la Paz et al., 2014; Sharma et al., 2019).

The mechanisms by which policosanols can exert their health effects are not fully understood, but Sharma et al. (2019) reported the involvement of GPR120 and suggested that these fatty alcohols are oxidized and converted to their corresponding FA, which subsequently can function as signalling molecules. The fatty alcohols found in Calanus oil are, however, not policosanols, and the above-mentioned results cannot be directly extrapolated to any potential

health effects of eicosenol and docosenol, since the degree of unsaturation and carbon chain length are important factors determining their health-providing potential.

Calanus oil is not the only natural source of eicosenols and docosenols. These fatty alcohols are also found as wax esters in the oil of certain deep sea fishes and bottle nose whales, as well as in the oil of jojoba (*Simmondsia californica*) seeds (Komori & Agawa, 1955; Miwa, 1971). Although these wax ester-rich oils have been mainly used for industrial purposes, a recent study from 2022, found that fatty alcohols in jojoba oil (11-eicosenol, 13-docosenol and 15-tetracosenol) had a cytotoxic effect in HEK293T cells at concentrations of 10 and 100 µM, but not at 1 µM (Acherki et al., 2022). The authors suggested that low concentration of these fatty alcohols could be used for treatment of topical transdermal or subdermal infections with enveloped viruses (e.g. herpes simplex virus) (Acherki et al., 2022; Verbiscar, 2005).

5.6 *Calanus finmarchicus* – a sustainable source of marine lipids

A few final words should be said regarding the sustainability of *Calanus finmarchicus* as a novel source of marine lipids. The consumption of seafood and the use of fish oil as a nutraceutical or in aquaculture feed, has increased over the past decades, and placed pressure on global fish stocks (FAO, 2022; Tocher, 2015). A recent FAO report stated that in 2019, 35% of the world's marine fisheries stocks were overfished, and this percentage has been increasing since 1974 (FAO, 2022). Yet there is a gap between supply and demand for marine oils, and thus a need for new and sustainable marine lipid sources (Tocher et al., 2019). *C. finmarchicus* and other lower tropic level organisms have been suggested as such as source. However, harvesting at the bottom of the food chain does not come without risk. Many ecologically and economically important fish species prey on *C. finmarchicus* (Prokopchuk & Sentyabov, 2006). Harvesting lower tropic levels species can thus result in deleterious consequences for planktivorous fish species and marine food webs if not done sustainably (Eysteinsson et al., 2018).

The proposed total catch quota for direct harvesting of *C. finmarchicus*, as set by the Norwegian Directorate of Fisheries, is 254 000 tonnes per year, while the actual harvest in 2016 was only 660 tonnes (Thorvik, 2017). This quota was set based on an estimated standing stock of 33 million tonnes in the Norwegian Sea, and followed similar regulations as for the harvest of krill (*Euphausia superba*) (Norwegian Directorate of Fisheries, 2016a, 2016b). It is argued that the current standing stock of *C. finmarchicus* is high enough to allow for an increase of the current quota without affecting the population size. Nevertheless, the total catch quota will be re-

assessed when new knowledge on the standing stock and harvesting activities are available (Fisheries, 2016a). It is therefore, currently safe to assume that *C. finmarchicus* is a sustainable source of marine lipids.

Box 1 The cardioprotective effect of EPA and DHA

Clinical studies with EPA and DHA have shown that these FA are able to reduce adipose tissue mass and system inflammation and to lower plasma lipid levels (Itariu et al., 2012; Skulas-Ray et al., 2019). The health effects of EPA and DHA with regard to cardiovascular diseases have, however, been controversial and inconclusive. A meta-analyses of 13 randomized control trials performed by Hu et al. (2019) concluded that marine omega-3 supplementation reduced the risk for coronary heart disease and cardiovascular disease. Later clinical trials, however, were not all able to reaffirm this conclusion. The ASCEND study from 2018 and the VITAL study from 2019 tested the effect of 1 g/day EPA + DHA on cardiovascular diseases in patients with diabetes and in the general population, respectively. Both clinical trials did not find any significant differences between the omega-3 supplemented group and the placebo group (Manson et al., 2019; The ASCEND Study Collaborative Group, 2018). The REDUCE-IT trial from 2019, however, did find a significant reduced risk in ischemic events and cardiovascular death with 4 g/day of purified EPA-ethyl ester supplementation in patients 45-years or older with established cardiovascular disease (Bhatt et al., 2019). The STRENGTH randomized clinical trial from 2020, on the other hand, also tested the effect of 4 g/day of EPA and DHA carboxylic acid, in patients with atherogenic dyslipidemia and high cardiovascular risk. They did find a 9% reduction in coronary heart disease in the EPA and DHA treated group. But not a significant difference in the outcome of major adverse cardiovascular events, except in people from Asian descent (Nicholls et al., 2020). The most recent clinical trial with EPA and DHA is the OMEMI trial, testing the effect of 1.9 g/day on elderly patients after myocardial infarction (Kalstad et al., 2021). Also this study could not detect a beneficial effect of EPA and DHA.

The discrepancies between the different results in clinical trials, and the countless observational studies that have shown an inverse correlation between omega-3 FA intake and the risk of ischemic heart disease, has led to confusion and questions regarding the use of marine oil supplements on the prevention of cardiovascular disease. Was the previously observed correlation between EPA and DHA and cardiovascular diseases due to other, cofounding, factors? Or are the different outcomes in clinical trials due to the study design of the trials? The former seems unlikely considering the vast number of observational studies documenting the health effects of marine lipids. Several things can, however, be said about the later question, regarding the study design of clinical trials (Farukhi et al., 2021).

Two striking differences between the five different clinical trials mentioned above, other than the populations studied, are the dosages of EPA and DHA and the choice of placebo oil. The ASCEND and VITAL studies both used olive oil as placebo (Manson et al., 2019; The ASCEND Study Collaborative Group, 2018). A Mediterranean diet, rich in olive oil, has, however, also been associated with a reduced risk to develop cardiovascular diseases (Delgado-Lista et al., 2016). The question therefore arises whether these two clinical trials studied the effect of EPA and DHA, or whether they compared the effect of these FA with that of oleic acid, the main constituent of olive oil. The REDUCE-IT study used a higher dose of omega-3 FA and used mineral oil as a placebo (Bhatt et al., 2019). This study has received criticism for their choice of placebo since mineral oil is believed to absorb the statins used by patients in the placebo group. The choice of placebo oil can thus potentially mask, or contribute, to the difference in outcome between the treatment group and the placebo group.

Due to the lipid lowering effect that omega-3 FA have (Skulas-Ray et al., 2019), the use of statins can also potentially mask the effect of these FA in the risk reduction to develop cardiovascular diseases. This becomes evident when the results of the STRENGHT study are compared with those of the JELIS trial from 2007 (Mitsuhiro et al., 2007). The patients included in the STRENGT study were at high risk for future cardiovascular events and background statin therapy was therefore required. The JELIS trial tested the effect of 1.8 g/day EPA in Japanese hypercholesterolaemia patients without using contemporary standards of care, and with a statin dose of only 10 mg/day pravastatin or 5 mg/day simvastatin. These dosages are considered “low-intensity statin therapy” according to the protocol used in the STRENGTH study (Nicholls et al., 2020). Unlike the STRENGTH study, where higher statin and omega-3 FA dosages were used, the JELIS study did find a significant reduction in the risk for major coronary events in the EPA treated group, compared to the control group (Mitsuhiro et al., 2007). A cofounding effect could however also be that patients from Asian descent are more susceptible to the positive health effects of omega-3 FA(Mitsuhiro et al., 2007; Nicholls et al., 2020).

A final focus point regarding the design of clinical trials are baseline values of the participants. The OMEMI trail studied the effect of EPA and DHA is patients living in Norway. The consumption of fish and seafood in Scandinavian countries is, however, considered to be higher than average. This could mean that baseline values of EPA and DHA are higher than average, and that any additional supplementation might not be able to result in an addition health effect. All in all, there are several aspects that need to be considered when studying and interpreting the results of clinical trials regarding the cardioprotective effects of omega-3 FA. Placebo, dose, statin use, and baseline values are only a few (Farukhi et al., 2021)

6 Conclusions and considerations for future studies

In this thesis we have shown that Calanus oil and its wax ester-derived fatty acids can potentially be used as a therapeutic approach to target obesity-induced metabolic distortions through its effect on the microbiome and on palmitate-induced cardiac lipotoxicity. The effect on the microbiome was studied using mice given a high-fat diet supplemented with 2% crude Calanus oil. The effect on lipotoxicity was studied in H9c2 cardiomyoblasts with the isolated Calanus oil wax esters-derived fatty acids.

As mentioned under “Research plan - challenges and obstacles”, we were unable to carry out the majority of the initially planned studies of this PhD project, e.g. the effect of Calanus oil on mitochondrial respiratory capacity in energy-consuming tissues, and fat mobilization and deposition in adipose tissue. In light of the previously observed anti-obesogenic action of the oil, we think that following up on these findings could still be considered for future studies. The method developed during my thesis, allowing the separation of Calanus oil wax ester-derived fatty acids and fatty alcohols, has provided us with useful experimental tools for future mechanistic studies regarding the selective effects of these compounds (both *in vivo* and *in vitro*) on the metabolic regulation and interplay between adipose tissue, liver, and muscle (including the heart). One idea is to examine whether Calanus oil or its constituents might cause browning of adipose tissue through activation of GPR120. In addition, it will be interesting to find out if the long-chain monounsaturated fatty alcohols function as signalling molecules on their own, or if they are oxidized to their corresponding long-chain monounsaturated fatty acids.

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Paper I

Possible Health Effects of a Wax Ester Rich Marine Oil [Review]

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REVIEW
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Possible Health Effects of a Wax Ester Rich Marine Oil

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The consumption of seafood and the use of fish oil for the production of nutraceuticals and fish feed have increased over the past decades due the high content of long-chain polyunsaturated omega-3 fatty acids. This increase has put pressure on the sustainability of fisheries. One way to overcome the limited supply of fish oil is to harvest lower in the marine food web. *Calanus finmarchicus,* feeding on phytoplankton, is a small copepod constituting a considerable biomass in the North Atlantic and is a novel source of omega-3 fatty acids. The oil is, however, different from other commercial marine oils in terms of chemistry and, possibly, bioactivity since it contains wax esters. Wax esters are fatty acids that are esterified with alcohols. In addition to the long-chain polyunsaturated omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the oil is also rich in stearidonic acid (SDA), long-chain monounsaturated fatty acids, and the long-chain fatty alcohols eicosenol and docosenol. Recent animal studies have indicated anti-inflammatory and anti-obesogenic actions of this copepod oil beyond that provided by EPA and DHA. This review will discuss potential mechanisms behind these beneficial effects of the oil, focusing on the impact of the various components of the oil. The health effects of EPA and DHA are well recognized, whereas long-chain monounsaturated fatty acids and long-chain fatty alcohols have to a large degree been overlooked in relation to human health. Recently, however the fatty alcohols have received interest as potential targets for improved health *via* conversion to their corresponding fatty acids. Together, the different lipid components of the oil from *C. finmarchicus* may have potential as nutraceuticals for reducing obesity and obesity-related metabolic disorders.

Keywords: *Calanus finmarchicus*, obesity, long-chain omega-3 fatty acids, long-chain monounsaturated fatty acids, long-chain fatty alcohol, stearidonic acid, cardiovascular diseases, inflammation

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; *Cf, Calanus finmarchicus;* DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, Eicosapentaenoic acid; GPR120, G-protein coupled receptor 120; HDL-C, high-density lipoprotein cholesterol; LC-MUFA, long-chain monounsaturated fatty acids; n-3 LC-PUFA, Omega-3 long-chain polyunsaturated fatty acids; LDL-C, low-density lipoprotein cholesterol; NF-κB, nuclear factor kappa B; PPAR, peroxisome proliferator-activated receptor; SDA, stearidonic acid; SFA: saturated fatty acid; TAG, triacylglycerol; TC, total cholesterol; TLR4, Toll-like receptor 4.

INTRODUCTION

It is widely accepted that the omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), present in seafood, have health benefits in several human diseases and conditions, such as cardiovascular and inflammatory diseases. They also play a critical role in neural development (Campoy et al., 2012; Delgado-Lista et al., 2012; Calder, 2015). Responsible organizations, such as the World Health Organization, European Food Safety Authority and the American Heart Association, recommend, therefore, at least one or two servings of (oily) fish per week, equivalent to about 250 mg/day of EPA and DHA (WHO, 2003; EFSA, 2009; Rimm et al., 2018).

The use of fish oil in aquaculture feed and as a nutraceutical for direct human consumption has increased over the past decades (Tocher, 2015). This, in addition to the increased consumption of seafood, has put pressure on sustainable fisheries, and it has been estimated that about 33% of the world's marine fish stocks are overfished (FAO, 2018). Still there is a gap between supply and demand for marine oils (Tocher, 2015; FAO, 2018) and therefore a need for new and sustainable sources of marine lipids. One possibility is to harvest lower in the marine food web. Zooplankton and small crustaceans like krill that feed on phytoplankton, the producers of the n-3 LC-PUFA, are to some degree now being utilized for production of marine oil nutraceuticals.

Calanus finmarchicus (*Cf*), a small marine copepod (**Figure 1**), constitutes a considerable proportion of the biomass in the Norwegian Sea (Planque and Batten, 2000) and is currently being harvested and industrially processed to an oil product, Calanus® Oil (Pedersen et al., 2014b). *Calanus finmarchicus* is an important prey item for many ecologically and economically important fish species such as herring and mackerel (Prokopchuk and Sentyabov, 2006), and harvesting lower down the food web can have serious impacts on the recruitment and survival of these planktivorous fish species if not done with care. Also, the by-catch of eggs, larvae, and fry during direct catching of *Cf* may have potential negative effects further up in the food chain (Eysteinsson et al., 2018). The Norwegian Directorate of Fisheries has proposed a total catch quota for direct catching of *Cf* of 254 000 tonnes a year (Norwegian Directorate of Fisheries, 2016a). This quota is based on an estimated standing stock of 33 million tonnes in the Norwegian Sea and following similar regulations as for krill (*Euphausia superba*) fishing in the Antarctic (Norwegian Directorate of Fisheries, 2016b). It is argued that the standing stock of *Cf* is so high that no effect of the proposed quota will be seen on the population size. In addition, although the proposed total quota is 254 000 tonnes a year, the actual harvest in 2016 was 660 tonnes (Thorvik, 2017). But nonetheless, the size of the total quota will be re-assessed at a later stage, when increased biological knowledge and more experience from the harvesting activities and catch processing on board is available (Norwegian Directorate of Fisheries, 2016a).

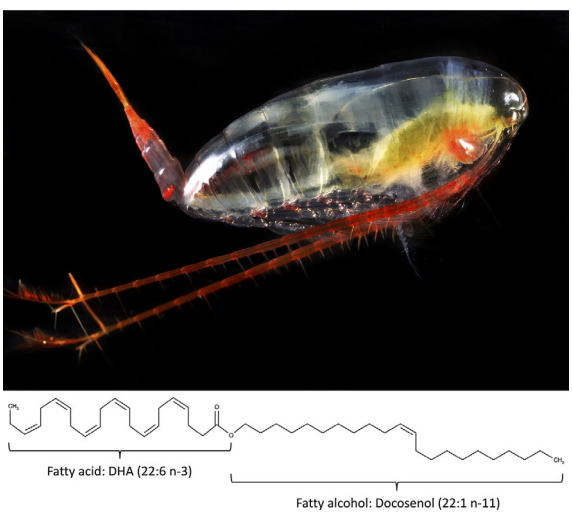

FIGURE 1 | The marine copepod *Calanus finmarchicus* and a wax ester composed of the long-chain polyunsaturated omega-3 fatty acid docosahexaenoic acid (22:6 n-3) bound to the long chain fatty alcohol docosenol (22:1 n-11) as an example of the lipids found in the oil of *Calanus finmarchicus*. Photo Copyright by Calanus AS.

C. finmarchicus has a lifespan of only one year (Falk-Petersen et al., 2009) resulting in very low levels of persistent organic pollutants in the lipid fraction (AMAP, 2009; Mizukawa et al., 2009), and refinement of the oil is therefore not necessary. The lipid class composition of the oil from *Cf* is, however, different from other marine oils. In traditional whole-body fish oil and cod liver oil, EPA and DHA are generally bound to a glycerol backbone forming triacylglycerol (TAG). Second-generation n-3 LC-PUFA fish oil supplements have concentrated contents of EPA and DHA, either in the form of ethyl esters or re-esterified TAG. Krill oil is also often included in this group, although it has a high content of phospholipids in addition to TAG (reviewed by Xie et al., 2019). The oil from *Cf* has a unique chemistry, where most of the fatty acids esterify with long-chain fatty alcohols, forming the lipid class known as wax esters (Lee et al., 2006) (**Figure 1**). For this reason, Calanus® Oil may be regarded as a third generation of n-3 products. This new marine oil is, however, a niche product compared to bulk oils, like fish oils. The total fish production globally in 2016 was around 171 million tonnes, of this approximately 20 million tonnes were used for non-food purposes, mostly for the production of fish meal and fish oil (FAO, 2018). This results in almost 1 million tonnes of fish oil (Tocher, 2015). In contrast, the harvest of *Cf* is below 1000 tonnes a year, and with a lipid content of approximately 8% (Falk-Petersen et al., 1987; Scott et al., 2000; Lee et al., 2006), this results in less than 100 tonnes of oil,

The aim of this review is to discuss the possible role of the novel marine oil from *Cf* as a metabolic therapy to prevent obesity-induced low-grade inflammation and the associated metabolic disturbances. However, a challenge with writing this review is the lack of clinical studies with calanus oil, while its

potential health benefits are based primarily on animal studies. The unique chemistry of this oil argues, however, for the view that this oil is not just another EPA- and DHA-containing oil. Therefore, we have chosen to discuss the impact of the major components of calanus oil on metabolic health in the light of available information in the literature.

BIOSYNTHESIS OF LONG-CHAIN OMEGA-3 FATTY ACIDS

Humans are not able to synthesize n-3 fatty acids *de novo* and, therefore, depend on the diet to obtain them directly, or synthesize them from dietary essential fatty acids such as α-linolenic acid (ALA). *De novo* synthesis of omega-6 and n-3 fatty acids from oleic acid is only possible in plants, including microalgae, because they possess the Δ-12 and Δ-15 desaturases. Delta-12 desaturase produces linoleic acid that can be converted further into ALA by Δ-15 desaturase. Humans can convert linoleic acid into arachidonic acid (AA) and ALA into SDA and EPA due to the enzymatic activity of Δ-6 and Δ-5 desaturases and elongase (**Figure 2**). The conversion of EPA to DHA is possible *via* two different pathways. After conversion of EPA into docosapentaenoic acid (DPA, 22:5n-3), further conversion into DHA can be done by the so-called Sprecher pathway. In this pathway DPA is first elongated (forming tetracosapentaenoic acid; 24:5n-3) followed by a second Δ-6 desaturation (forming tetracosahexaenoic acid; 24:6n-3) and finally chain shortening *via* peroxisomal β-oxidation to DHA (Sprecher et al., 1995). Lower eukaryotes and some vertebrates, even including some mammals, but not humans, can convert DPA directly into DHA by Δ-4 desaturase (**Figure 2**). The Δ-6 desaturase activity is rate-limiting (Bernert and Sprecher, 1975) making the conversion of ALA to SDA inefficient and the conversion further to DHA very limited. More EPA is therefore formed from SDA than from ALA, but it is only slightly further converted to DHA due to the second Δ-6 desaturase step (Leonard et al., 2004; Lee et al., 2016). The endogenous conversion of ALA to EPA and DHA has been reported to be 21% and 9%, respectively, in young women (Burdge and Wootton, 2002). In men, the conversion from ALA to EPA is only between 0.3% and 8% while the conversion from ALA to DHA is below 4% and often undetectable (Emken et al., 1994; Burdge et al., 2002; Burdge et al., 2003; Hussein et al., 2005). It is therefore important to consume EPA and DHA *via* the diet in order to benefit from the health effects provided by these fatty acids.

OIL EXTRACTED FROM *CALANUS FINMARCHICUS*

The composition of the different lipid classes in the oil in *Cf* changes depending on the copepod life-cycle stage at the time of harvest, season, and location of sampling (Falk-Petersen et al., 1987; Fraser et al., 1989) (see also **Tables 1** and **2**). The industrially produced oil is obtained in the summer when *C. finmarchicus* is in surface waters and has the highest lipid content due to feeding on the blooming phytoplankton (Pedersen et al., 2014b).Throughout its 1-year life cycle, the lipid content and fatty acid composition of *C. finmarchicus* changes, depending on the life stage/season (Kattner and Krause, 1987). In this Calanus® Oil, more than 85% of the lipids consist of wax esters (Pedersen et al., 2014a) (see also **Table 2**). About 11% of the fatty acids (all lipid classes combined) are monounsaturated fatty acids (MUFA), of which cetoleic acid

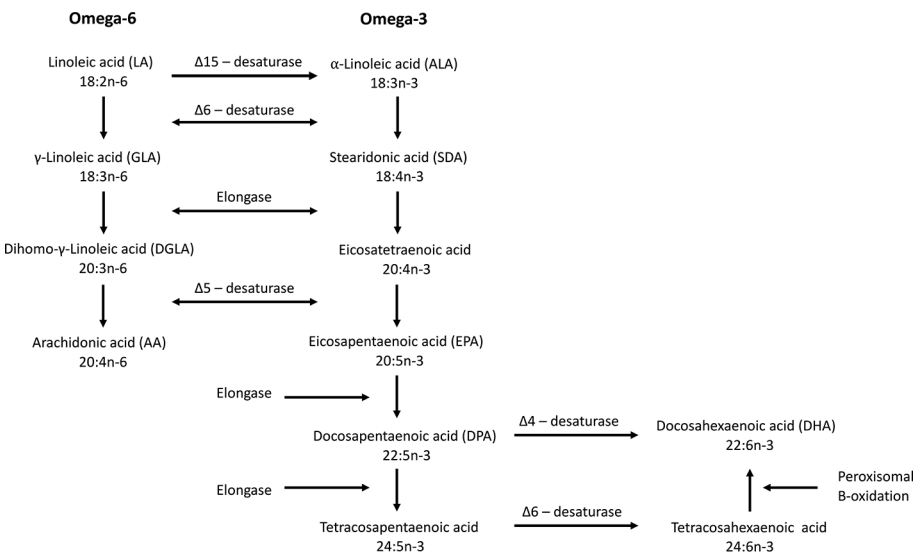

FIGURE 2 | Biosynthesis of omega-6 and omega-3 fatty acids. Δ-15 desaturase is only found in plants. Δ-4 desaturase is present in some vertebrates but not in humans. The Δ-6 desaturase catalyze is the rate limiting step in the synthesis of EPA and DHA (modified from Calder, 2015).

TABLE 1 | Lipid class composition of *C. finmarchicus* sampled in summer, autumn, winter, and spring in Balsfjord, Norway.

	% Total Lipid			
Lipid Class:	**June**	**October**	**January**	**March**
Wax esters	85.4	88.1	90.0	84.9
Phospholipids	4.2	7.3	5.7	10.3
Triacylglycerols	8.9	1.3	0.8	nd
Cholesterol	1.2	2.6	2.4	3.2
Free fatty acids	0.2	nd	1.1	1.7

nd, not detected. Source: Falk-Petersen et al. (1987).

TABLE 2 | Fatty acid and fatty alcohol composition (mass %) of the commercial Calanus® Oil and in the different lipid classes in *Calanus finmarchicus*.

	Lipid class				
Fatty acid	**Calanus® Oil[a]**	**WE[b]**	**WE[c]**	**TAG[b]**	**PL[b]**
14:0 (Myristic)	6.4	26.3	18.0	12	3.3
16:0 (Palmitic)	4.5	9.8	9.3	30.4	25.8
18:0 (Stearic)	0.2	0.9	nd	6.1	3.6
16:1n-7 (Palmitoleic)	1.7	6.7	6.5	3.6	1.1
18:1n-9 (Oleic)	1.6	5.3	5.3	10.4	2.5
20:1n-9 (Gondoic)	2.4	7.8	9.6	nd	0.2
22:1n-9 (Erucic)	0.3	0.2	nd	nd	nd
22:1n-11 (Cetoleic)	4.3	7.0	12.0	2.2	0.2
18:2n-6 (LA)	0.7	1.2	nd	2.7	1.5
18:3n-3 (ALA)	1.4	1.5	nd	2.3	0.6
18:4n-3 (SDA)	7.0	13.7	9.3	5.9	2.5
20:5n-3 (EPA)	5.5	11.4	9.8	8.7	19.2
22:5n-3 (DPA)	0.3	nd	nd	1.2	0.2
22:6n-3 (DHA)	3.9	2.2	7.7	5.8	37.4
Fatty alcohol					
14:0	0.4	3.9	1.1		
16:1n-7	0.5	3.4	1.8		
18:1n-9	1.0	nd	nd		
20:1n-9 (Eicosenol)	12.9	39.3	41.0		
22:1n-9	1.0	nd	nd		
22:1n-11 (Docosenol)	18.8	38.8	45.2		

Source: [a]Pedersen et al. (2014a), [b]Albers et al. (1996) [c]Kattner et al. (1989).
The fatty acid values per lipid class presented here are from C. finmarchicus females[b] and copepod stage V[c] harvested during the summer in the Fram Strait. Modified from Pedersen et al. (2014b) and Falk-Petersen et al. (2009).
nd, not detected; SFA, saturated fatty acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; WE, wax ester; TAG, triacylglycerol; PL, phospholipid.

(22:1n-11) and gondoic acid (20:1n-9) are the most abundant with about 4% and 2.5%, respectively, followed by 1.5% oleic acid (18:1n-9). Approximately 19% of the fatty acids are PUFA of which 18% are omega-3 fatty acids. This novel marine oil is relatively low in EPA and DHA compared to other marine oils (6% and 4% respectively), but is relatively rich in SDA (7%), see **Table 2** (Pedersen et al., 2014a; Cook et al., 2016b). The main fatty alcohols present are the equivalents of the dominant LC-MUFA in the oil, namely the monounsaturated long-chain fatty alcohols eicosenol (20:1n-9) and docosenol (22:1n-11) (Pedersen et al., 2014a). In **Table 2**, it can be seen that in oil in *C. finmarchicus* the abundance of EPA and DHA is highest in the phospholipids. The actual amount, however, is low since the proportion of phospholipids is low compared to the neutral lipid classes (**Table 1**). It has been published that the bioavailability of EPA and DHA is higher from the phospholipids than from TAG (Maki et al., 2009; Schuchardt et al., 2011; Ulven and Christiansen, 2015; Cook et al., 2016a). But this remains, however, controversial (Salem and Kuratko, 2014). Phospholipids are, however, not detected in the commercial Calanus® Oil (Pedersen et al., 2014a) probably due to endogenous enzymatic hydrolysis (Vang et al., 2013). The oil has also been reported to contain about 1500 ppm astaxanthin (Pedersen et al., 2014b). This antioxidant has anti-inflammatory and anti-atherogenic potential, which has been extensively studied in both humans and animals (Jacobsson et al., 1999; Jacobsson et al., 2004; Pashkow et al., 2008; Yang et al., 2011a). However, any possible health effects of astaxanthin will not be discussed here.

A complete description of the digestion of wax esters and absorption of fatty acids and fatty alcohols are beyond the scope of this paper. But minor amounts of waxes are present in a variety of food items (Hargrove et al., 2004). The consumption of large portions of wax ester rich fish has been reported to cause outbreaks of keriorrhea "oily diarrhea," and associated stomach cramps, nausea, and vomiting, in several countries. This has led to the suggestion that wax esters are indigestible (Ho Ling et al., 2009). However, other publications have demonstrated that mammals can digest wax esters, at least when consumed in moderate amounts (Hansen and Mead, 1965; Yaron et al., 1982; Gorreta et al., 2002) As reviewed by Hargrove et al. (2004), humans are able to hydrolyze the waxes found in a variety of food items and absorb the liberated fatty acids and alcohols. The safety of the oil from *Cf* for human consumption has been clinically evaluated by Tande et al. (2016), and there are no safety concerns regarding this novel marine oil when consumed in recommended amounts of 2g of calanus oil. Parallel to this safety trial ran a study on the bioavailability of EPA and DHA in oil from *Cf* for human consumption (Cook et al., 2016b). The volunteers in the study by Cook et al. (2016b) consumed 4g calanus oil without any ill effects.

Feeding experiments in mice have shown that the fatty alcohols present in the oil are detected in the feces of mice, indicating that the wax esters are indeed hydrolyzed. The same study also detected increased incorporation of different n-3 LC-PUFA in liver and white adipose tissue, indicating absorption of wax ester-derived fatty acids (Pedersen et al., 2014a).

Calanus oil is a novel marine oil, and it has only since recently been harvested for production of a nutraceutical. It has earlier been used in feed for farmed Atlantic salmon (*Salmo salar*) (Bogevik et al., 2009) and Atlantic halibut (*Hippoglossus hippoglossus*) (Colombo-Hixson et al., 2013). However, oils from zooplankton are considerably more expensive than fish oils and are, therefore, currently not included in general aquaculture feed. Clinical studies are currently being conducted to examine the ability of oil from *Cf* to combat obesity and insulin resistance, but no results have been released to date. Feeding experiments on rodents, however, have shown that dietary supplementation with only 1% to 2% calanus oil improved metabolic and inflammatory parameters in

high-fat diet-induced obese mice (Höper et al., 2013; Höper et al., 2014). The oil has also been reported to attenuate atherosclerotic lesion formation (Eilertsen et al., 2012), reduce hypertension (Salma et al., 2016), and protect the heart from ischemic stress (Jansen et al., 2019). Results from Höper et al. (2014) indicated that supplementation of the diet with purified wax ester has stronger anti-inflammatory and anti-obesogenic effects in diet-induced obese mice, compared to ethyl esters of EPA and DHA. This suggests that not only EPA and DHA, but also other components from the hydrolyzed wax esters in the oil, or the wax ester itself, might have beneficial effects on health. In particular, the observation that even very low dosages of the oil can counteract obesity-induced metabolic dysfunction holds promise that calanus oil could be promising nutraceutical in the future

OBESITY INDUCED CHRONIC LOW-GRADE INFLAMMATION

Obesity, in particular abdominal obesity, is associated with a chronic local low-grade inflammation (Solinas and Karin, 2010; Gregor and Hotamisligil, 2011; Ouchi et al., 2011) with progressive immune cell infiltration in adipose tissue (**Figure 3**). In this process, the immune cells and (to a lesser extend) the enlarged/expanded adipocytes start to secrete pro-inflammatory cytokines (e.g., TNFα, IL-6, and IL-1β) and chemokines, such as monocyte chemoattractant protein-1 (MCP-1) (Fain, 2006).

Numerous studies have shown that hypoxia and nutrient excess are the two main triggering factors for inflammation in adipose tissue (Schenk et al., 2008; Ye, 2009; Gregor and Hotamisligil, 2011). In response to nutrient excess, adipocytes expand and become hypertrophic. At the same time, the distances between the blood vessels increase and oxygen diffusion becomes insufficient (Torres Filho et al., 1994), leading to local hypoxia, which in turn triggers the secretion of cytokines *via* activation of Hypoxia-Inducible Factor (HIF)-1 Alpha (Sun et al., 2013).

Infiltration of pro-inflammatory cells in inflamed adipose tissue is characterized by infiltration of M1 macrophages in replacement of M2 macrophages. M2 macrophages produce anti-inflammatory cytokines such as IL-10 and IL-23 and recruit regulatory T cells. M1 macrophages, however produce pro-inflammatory cytokines such as TNFα and IL-6, and attract Th1 cells (Fujisaka et al., 2009; as reviewed in Mills, 2012). Adipose tissue is the key site of interaction between adipocytes and immune cells due to the architectural organization and proximity of these cell types. Access to blood vessels allows for soluble mediators to communicate with other organs. In this way, the inflammatory status of adipose tissue becomes a risk factor for disease development, including metabolic syndrome, insulin resistance, diabetes mellitus, and cardiovascular disease (Mokdad et al., 2003; Reaven, 2005; Hotamisligil, 2006; Mathew et al., 2008; Van de Voorde et al., 2013) (**Figure 3**).

THE MAIN COMPONENTS OF THE OIL FROM *CF* AND THEIR EFFECT ON INFLAMMATION CONTROL

EPA and DHA

Fish oils have long been considered to promote positive health effects through the n-3 LC-PUFA EPA and DHA (Simopoulos, 1991). Treatment of severely obese non-diabetic patients with EPA and DHA has shown to reduce adipose tissue mass and systemic inflammation (Itariu et al., 2012). An updated meta-analysis of 13 randomized controlled trials, which included over 120 000 participants confirmed that marine n-3 LC-PUFA supplementation reduces the risk for coronary heart disease (CHD) and cardiovascular disease (CVD), myocardial infarction, and death due to CHD and CVD (Rimm et al., 2018; Hu et al., 2019). The American Heart Association concluded, based on new scientific data, that the prescription of n-3 LC-PUFA at a dose of 4 g/day can be used as monotherapy or in combination with other lipid-lowering agents to reduce hypertriglyceridemia (Skulas-Ray et al., 2019). Clinical studies and a recent meta-analysis, including 20 clinical trials, have confirmed therapeutic effects of n-3 LC-PUFA supplementation in rheumatoid arthritis patients (Gioxari et al., 2018; Woodman et al., 2019). Moreover, a systematic review and meta-analysis by Natto et al. (2019) concluded that these omega-3 fatty acids may be associated with lower plasma levels of inflammatory biomarkers in diabetic patients. However, results regarding their effect on glucose metabolism, insulin resistance, and type 2 diabetes are less clear (Stella et al., 2018). Also regarding other chronic diseases, such as non-alcoholic fatty liver disease and chronic kidney disease, the effects of n-3 LC-PUFA are inconclusive (Jump et al., 2018; Saglimbene et al., 2019). Factors that may account for the inconsistent findings regarding the use of n-3 LC-PUFA supplements are the doses used, the choice of placebo, and the duration and type of intervention (El-Bayoumy and Manni, 2020).

Although the benefits of consuming n-3 LC-PUFA may remain controversial for some diseases and conditions, it is well accepted that n-3 LC-PUFA have anti-inflammatory effects. These anti-inflammatory effects and their possible mechanisms have been extensively reviewed by Calder (2015) and Rogero and Calder (2018). The two main mechanisms are changes in the phospholipid composition of the cell membrane and changes in the activation of pro- and anti-inflammatory transcription factors (**Figure 4**) and their target genes.

Alterations of the Membrane Phospholipid Composition of Immune Cells

Intake of n-3 LC-PUFA alters the fatty acid composition of the membrane phospholipids of immune cells, affecting the production of AA- and EPA-derived eicosanoids from the phospholipids. AA is a major substrate for the enzymes cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450. Enzymes that catalyze the metabolisms of AA to 2-series prostaglandins and thromboxanes and 4-series leukotrienes and

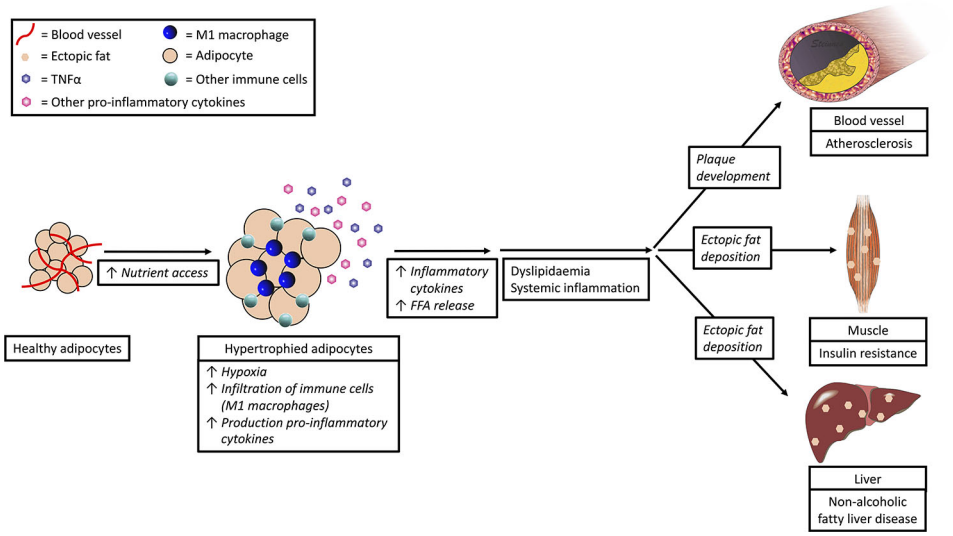

FIGURE 3 | Expansion of adipocytes due to nutrient access leads to dyslipidemia and inflammation and subsequent cardiovascular disease, insulin resistance, and non-alcoholic fatty liver disease.

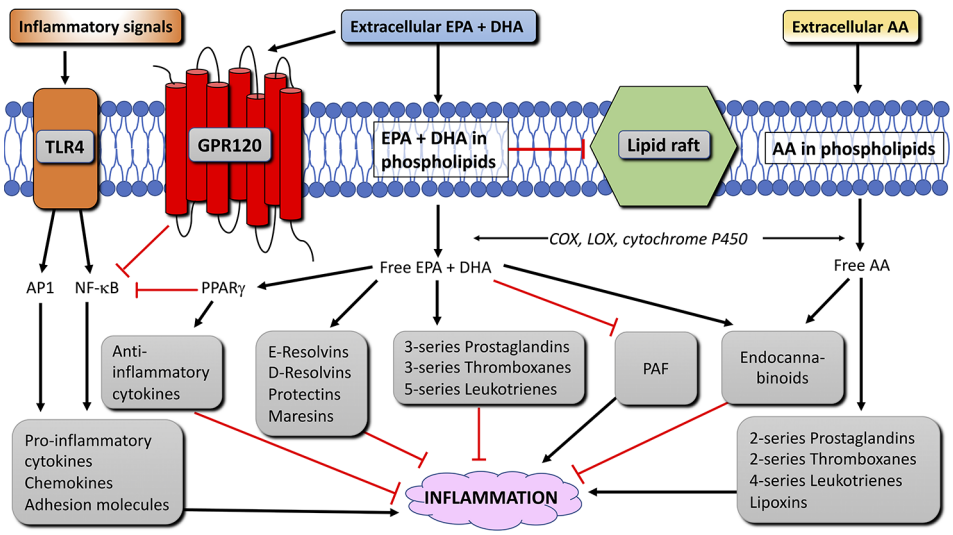

FIGURE 4 | Anti-inflammatory mechanisms and pathways of EPA and DHA in immune cells. Black arrow lines indicate activation, red blunted lines indicate inhibition. AA, arachidonic acid; AP1, activation protein 1; COX, cyclooxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GPR120, G-protein receptor 120; LOX, lipoxygenase; NFκB, nuclear factor κ B; PAF, platelet-activating factor; PPAR, peroxisome proliferator activated receptor; TLR4, toll-like receptor 4 (modified from Calder, 2015).

lipoxins that mainly serve as pro-inflammatory lipid mediators (reviewed in Calder, 2009). EPA is a substrate for the same enzymes, and competes with AA as a substrate for COX and LOX. EPA-derived eicosanoids form 3-series prostaglandins and thromboxanes and 5-series leukotrienes. These EPA-derived eicosanoids have been regarded less potent than those produced from AA, and thereby stimulate inflammation to a lesser extent (Goldman et al., 1983; Lee et al., 1984; Bagga et al., 2003; Wada et al., 2007; Tull et al., 2009). Calder (2009), pointed out, however, that not all AA-derived eicosanoids (e.g. prostaglandin E2) are pro-inflammatory.

Production of Lipid Mediators That Resolve Inflammation

The incorporation of n-3 LC-PUFA in the cell membrane increases the production of lipid mediators that resolve inflammation, namely, the EPA-derived E-series resolvins and the DHA-derived D-series resolvins, protectins, and maresins

(**Figure 4**). These lipid mediators were shown to have anti-inflammatory and protective properties both in cell culture and animal models of inflammatory diseases, and aid in the resolution of the inflammation (2008a; Serhan et al., 2008b; Bannenberg and Serhan, 2010; Serhan and Chiang, 2013; Balas et al., 2014). In addition to eicosanoids and pro-resolving lipid mediators, phospholipids containing n-3 (and n-6) LC-PUFA can also become metabolized to produce endocannabinoids. The endocannabinoids can bind to cannabinoid receptor types 1 and 2, which have anti-inflammatory properties (Artmann et al., 2008; Batetta et al., 2009; Wood et al., 2010). Other studies have shown that through incorporation in the cell membrane, n-3 LC-PUFA reduce the production of platelet-activating factor in certain immune cells (Croft et al., 1986; Pickett et al., 1986; Sperling et al., 1987; Shikano et al., 1993; Martin–Chouly et al., 2000; Watanabe et al., 2001). Finally, incorporation of EPA and DHA disrupts the formation of lipid rafts in the membrane, which can lead to changes in cell signal transduction during inflammation (Reviewed by Pike, 2003).

Long-Chain Omega-3 Fatty Acid-Induced Activation of Transcription Factors

Nuclear factor kappa B (NF-κB) is a transcription factor that up-regulates genes encoding pro-inflammatory cytokines, adhesion molecules, chemo-attractants, and enzymes needed to produce eicosanoids. Toll-like receptor 4 (TLR4) is a membrane protein that, upon activation, initiates a signaling pathway that activates NF-κB and the transcription factor activator protein 1 (AP1), leading to increased inflammation (**Figure 4**). In adipose tissue of obese people, TLR4 signaling triggers chronic low-intensity inflammation (reviewed in Rogero and Calder, 2018). Long-chain omega-3 fatty acids, however, limit the activation of TLR4 by inhibiting the translocation of TLR4 to lipid rafts, due to the disrupting effect these fatty acids have on raft formation (Rogero and Calder, 2018). Long-chain omega-3 fatty acids further reduce the inflammatory effect of NF-κB and AP1 by binding to G-protein coupled receptor 120 (GPR120) (Oh et al., 2010; Oh and Olefsky, 2012). GPR120 is highly expressed in adipocytes, the distal part of the intestine, and in macrophages (Gotoh et al., 2007; Oh et al., 2010). GPR120 activation in macrophages inhibits activation of NF-κB and thereby reduces inflammation (Oh et al., 2010; Li et al., 2013; Yan et al., 2013; Williams-Bey et al., 2014) (**Figure 4**). Peroxisome proliferator-activated receptor (PPAR)-γ is a transcription factor expressed in immune cells and adipocytes. PPARγ can be activated by omega-3 fatty acids and physically interfere with NF-κB and thereby decreasing inflammation (**Figure 4**). Furthermore, PPARγ can form a heterodimer with retinoid-X-receptor (RXR). Both PPAR-γ:RXR as well as PPARγ and RXR alone are transcription factors for anti-inflammatory mediators and can be activated by PUFA and lipid mediators produced from AA, EPA, and DHA (Forman et al., 1997; Kliewer et al., 1997; de Urquiza et al., 2000; Desreumaux et al., 2001; Vanden Berghe et al., 2003; Szanto and Nagy, 2008; Zapata-Gonzalez et al., 2008). Finally, due to the disruption of membrane rafts and its associated intracellular signaling by EPA and DHA, n-3 LC-PUFA also inhibit T cell responses (Stulnig and Zeyda, 2004; Yaqoob, 2009; Kim et al., 2010). Thus, EPA and DHA have anti-inflammatory effects *via* several mechanisms. The interaction between these fatty acids and other cellular components in immune cells is illustrated in **Figure 4**.

To sum up, it appears that n-3 LC-PUFA can attenuate diet-induced obesity and inflammation *via* several mechanisms. In light of the fact that calanus oil contains relatively small amounts of these particular fatty acids, it is likely that it is not only EPA and DHA which are responsible for the anti-obesogenic and anti-inflammatory effects of the oil, but other components in addition. Of note, these effects were obtained with a low supply (1.5%) of calanus oil (Höper et al., 2013; Höper et al., 2014), whereas Mori et al. (2007) reported reduced adiposity in mice fed a high-fat diet supplemented with fish oil (containing 44.8% DHA and 5.9% EPA) only when the content of fish oil reached 8%.

Stearidonic Acid

SDA is the Δ-6 desaturation product of ALA (**Figure 2**). Oil from *Cf* contains about 7% SDA (Pedersen et al., 2014a; Cook et al., 2016b), which is high compared to fish oils (McGill and Moffat, 1992). SDA is naturally present at about 12% in echium oil and at 20% in *Buglossoides arvensis* oil, also called Corn gromwell (Bimbo et al., 2013). In addition, it has now been developed gene-modified soybeans in which SDA is enriched. The SDA content in such soybean oil is about 20% (Wilkes, 2008). The main effect of SDA on inflammation control appears to be dependent on its conversion to EPA, while little is known about any direct effects. Supplementation with SDA-containing oil to the diet increases the abundance of EPA in blood lipids, peripheral blood mononuclear cell, other immune cells, red blood cells, and in heart tissue in both humans and animals (James et al., 2003; Miles et al., 2004a; Miles et al., 2004b; Harris et al., 2007).

Although the EPA concentration is often increased upon SDA consumption, any subsequent health effects are not clear (Banz et al., 2012; Deckelbaum et al., 2012; Whelan et al., 2012; Walker et al., 2013). SDA supplementation trials with overweight or slightly obese human volunteers have shown inconclusive results regarding the effect on the omega-3 index, which may be an important measure for the risk of developing CVD and insulin resistance (Burrows et al., 2011). Pieters and Mensink (2015) did not find any effect of SDA on the omega-3 index despite an increase in EPA in the red blood cell membranes, whereas other studies did confirm an increase in the omega-3 index (Harris et al., 2008; Lemke et al., 2010). Supplementation of SDA-rich oil does not appear to have an effect on TAG, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) concentrations in plasma of healthy, overweight or slightly obese humans (Harris et al., 2008; Whelan, 2009; Lemke et al., 2010; Krul et al., 2012; Pieters and Mensink, 2015). However, the lipid profile was improved in lean and obese Zucker rats fed SDA-enriched soybean oil (Casey et al., 2013).

Also, regarding inflammation, the health benefits of SDA supplementation remain unclear. There was no effect on the

production of TNFα and IL-1β in LPS-stimulated whole blood from healthy volunteers (James et al., 2003). Miles et al. (2004b) observed that SDA can increase the EPA status in immune cells, but did not observe an effect on human immune function. While Hsueh et al. (2011) found a reduced IL-6 secretion in LPS-stimulated adipose stem cells from *ob/ob* mice due to a suppressed TLR2 expression and a decreased activity of NF-κB. SDA also downregulated the levels of inducible nitric oxide synthesis (iNOS) protein, the translocation of NF-κB and the phosphorylation of mitogen-activated protein kinases (MAPK) in LPS-induced (M1) macrophages (Sung et al., 2017).

SDA does not increase the abundance of DHA in humans, due to the second Δ-6 desaturation step (**Figure 2**). This might be one of the reasons why SDA does not appear to have a clear effect on human health. Other reasons can be the low doses used or the duration of the experiment. Also, as with EPA and DHA, the chemical structure (TAG, ethyl ester or wax ester) might play a role.

Although the effect of SDA on immune function and lipid profile remains inconclusive, SDA is one of the most potent fatty acids for activating GPR120 (Kotarsky et al., 2003; Christiansen et al., 2015). GPR120 is highly expressed in macrophages and adipocytes. The two cell types that play a crucial role in obesity and the development of the underlying chronic inflammation and metabolic syndrome. As previously stated, GPR120 activation in macrophages has anti-inflammatory effects. GPR120 activation in adipocytes stimulates adipocyte differentiation (Gotoh et al., 2007; Miyauchi et al., 2009) and improves insulin sensitivity and enhances glucose uptake due to an increased translocation of glucose transporter 4 (GLUT4) from the cytosol to the cell membrane (Talukdar et al., 2011). In murine models, the expression of GPR120 in adipose tissue is induced by thermogenic activation, promoting browning of white adipose tissue and brown fat activation. Browning of white adipose tissue is an important component of energy expenditure and can lead to weight loss (Quesada-López et al., 2016; Sharma et al., 2019). GPR120 activation is reported to improve glucose tolerance, insulin resistance, and chronic inflammation in obese mice and is, therefore, a target for the treatment of obesity and type 2 diabetes (Oh et al., 2010; Ichimura et al., 2012; Oh et al., 2014; Yore et al., 2014).

The diverse tissue distribution of the GPR120 may indicate several functions related to systemic metabolism and inflammation. Recently, more attention has been given to the anti-inflammatory role of this receptor in intestinal cells (Anbazhagan et al., 2016). It has been shown that the expression of GPR120 and other free fatty acid receptors (GPR40 and GPR119) are more abundant in the lower intestine, especially in the colon (Hirasawa et al., 2005; Miyauchi et al., 2009; van der Wielen et al., 2014). However, dietary lipids, such as TAG and phospholipids are quickly digested and absorbed in the upper parts of the gastrointestinal system and will normally not reach the lower intestine (Carey et al., 1983). In contrast, wax esters in calanus oil are more hydrophobic than dietary TAG and, therefore, more difficult to emulsify. In addition, other enzymes than those hydrolyzing TAG and phospholipids are probably involved. As a result, wax esters may exhibit a longer retention time to facilitate hydrolysis and absorption (Cowey and Sargent, 1977; Verschuren and Nugteren, 1989). The wax esters may therefore act as a natural delayed release of potent stimulators of GPR120, such as SDA, EPA, and DHA in this part of the intestinal system. Of interest, ongoing studies aim at developing systems of nutrient delivery to pass beyond the proximal small intestine by using a form of enteric coating to obtain a delayed release of the potent GPR120 agonists (Sørensen, 2018).

The health benefit of SDA is not clear, but the mechanism may partly depend on its conversion to EPA, so that it acts indirectly *via* the mechanisms described in section *EPA and DHA*. In addition, the ability of SDA to activate GPR120 receptors on macrophages, adipocytes, and intestinal cells could be another mechanism by which SDA could alleviate obesity-induced inflammation. Both mechanisms seem plausible in light of the high content of SDA in calanus oil and should be followed up by new mechanistic studies.

Monounsaturated Fatty Acids

About 10% of the fatty acids in calanus oil are monounsaturated fatty acids, of which cetoleic acid (22:1 n-11) and gondoic acid (20:1 n-9) are the most abundant, with approximately 4% and 2.5%, respectively (Pedersen et al., 2014a; Cook et al., 2016b). The interest for monounsaturated fatty acids in a health perspective is mainly based on the observation that the incidence of chronic diseases is relatively low among the adult population in certain regions bordering the Mediterranean Sea. Olive oil, which is a major component of the Mediterranean diet, is rich in oleic acid (18:1n-9), and the health-promoting effect of the diet has to some extent been ascribed to this MUFA (Delgado-Lista et al., 2016). Dietary MUFA have been associated with cardio protection (Pérez-Jiménez et al., 2002) and reduction of risk factors for development of metabolic syndrome (reviewed in by Gillingham et al., 2011). The replacement of saturated fatty acid (SFA) with MUFA (18:1n-9) in the diet may improve the blood lipid profile by lowering TAG, TC, and (V)LDL-C, while preserving HDL-C. In addition, replacement of SFA with MUFA improves body composition and insulin sensitivity while reducing hyperglycemia and hypertension in individuals predisposed to metabolic syndrome (reviewed in Gillingham et al., 2011).

More recently, the role of long-chain mono-unsaturated fatty acids (LC-MUFA), having aliphatic chains of more than 18 C atoms, has been studied (reviewed by Yang et al., 2016b). These fatty acids are found in high amounts in many fish oils (McGill and Moffat, 1992). Oil from *Cf* is rich in the LC-MUFA, cetoleic acid (22:1n-11), and a recent report indicated that this particular fatty acid may improve the efficiency of the conversion of ALA to EPA and DHA (Østbye et al., 2019).

Dietary intake of different marine oils rich in LC-MUFA (in addition to n-3 LC-PUFA) reduces the risk factors of metabolic syndrome in animal models by improving plasma lipid levels and insulin sensitivity (Østerud et al., 1995; Lindqvist et al., 2009; Yang et al., 2011b; Yang et al., 2011a; Yang et al., 2015). Saury

and herring oil have been reported to decrease adipocyte size and cause an increase in n-3 LC-PUFA levels and a concomitant decrease in n-6/n-3 PUFA ratio in different tissues (Lindqvist et al., 2009; Yang et al., 2011b; Yang et al., 2015). Herring oil, and seal oil combined with olive oil, have been found to reduce atherosclerotic lesions in the aorta (Eilertsen et al., 2011; Gabrielsson et al., 2011). Saury oil is also reported to reduce hepatic TC (Yang et al., 2011c) and TAG content (Yang et al., 2011c; Yang et al., 2015). Furthermore, saury and pollock oil increased plasma adiponectin levels, and decreased plasma levels of resistin, leptin, (Yang et al., 2011c; Yang et al., 2011b) and TNFα (Yang et al., 2011b) in animal models.

The marine oils used in the studies mentioned above are all rich in LC-MUFA. However, they also contain n-3 LC-PUFA, and it is therefore not possible to attribute the observed biological effects solely to the LC-MUFA component in the various oil preparations. A few studies have, however, used diets supplemented with concentrated LC-MUFA to investigate the effects attributed specifically to this class of fatty acids. This type of experimental design has investigated the health effects of LC-MUFA on atherogenesis, obesity-induced inflammation, glucose and lipid metabolism, and the expression of associated genes in different animal models (Halvorsen et al., 2001; Yang et al., 2011d; Yang et al., 2013; Yang et al., 2015; Yang et al., 2016a; Yang et al., 2017). From these studies, it can be concluded that LC-MUFA decrease atherosclerotic lesion formation, reduce cholesterol efflux and alter gene expression related to inflammation, lipid metabolism and energy expenditure in different tissues (**Figure 5**). It appears that the carbon chain length of the dietary MUFA can be an important factor that determines its metabolic effects. For instance, LDL receptor knock out (LDLR-KO) mice fed a Western diet enriched with 2% LC-MUFA concentrate displayed suppressed levels of aorta atherosclerotic lesions and plasma inflammatory markers such as C-reactive protein (CRP), macrophage-colony stimulating factor (MCSF) and complement component 1q, receptor 1(C1qR1). These effects were not observed when the mice were fed the same diet enriched with 2% oleic acid-rich olive oil when compared to control (Yang et al., 2016a).

Although chain length might be an important factor, the exact mechanisms behind the health promoting and cardioprotective effects of LC-MUFA are not fully understood. However, LC-MUFA are considered ligands of PPARs (Grygiel-Górniak, 2014). Thus, LC-MUFA concentrate supplementation has been shown to increase the expression of *pparγ* and its target genes, and decreased inflammatory marker expression in white adipose tissue (Yang et al., 2013). This was associated with reduced adipocyte size. LC-MUFA have also been reported to decrease atherosclerosis *via* PPAR signaling (Yang et al., 2016a; Yang et al., 2017). *Pparα*, *pparγ*, and their target genes *Cyp7a1* (encoding cholesterol 7α-hydroxylase or bile acid synthase) and *Adipor2* (gene for adiponectin 2 receptor) were reported to be upregulated in the liver of the LC-MUFA fed mice (Yang et al., 2016a). Over-expression of the CYP7A1 enzyme protects against atherosclerosis (Miyake et al., 2002) and adiponectin improves metabolic syndrome and atherosclerosis (Hui et al., 2012).

As mentioned, other studies using concentrated LC-MUFA (Yang et al., 2011d) or LC-MUFA rich fish oil (Yang et al., 2011b; Yang et al., 2011c; Yang et al., 2015) have reported that these fatty acids stimulate expression of other genes involved in inflammation, lipid metabolism and insulin signaling (see also **Figure 5**). However, the direct involvement of the PPAR signaling pathway in the expression of each of these genes is not fully described. Further studies are needed to elucidate if LC-MUFA exert beneficial health effects *via* mechanisms other than the proposed PPAR signaling pathways, and if some of these direct mechanisms distinguish the effects of LC-MUFA from those of LC n-3 PUFA.

Fatty Alcohols

The most abundant fatty alcohols in calanus oil are the monounsaturated fatty alcohols docosenol (22:1 n-11) and eicosenol (20:1 n-9) (**Table 2**). The health promoting properties of fatty alcohols became an area of interest when researchers in Cuba (Mas et al., 1999; Castaño et al., 2001; Arruzazabala et al., 2002) reported beneficial effects of policosanol from sugarcane wax on the plasma lipoprotein profile (increased HDL-C and reduced TC and LDL-C). Policosanol is a mixture of essential very-long-chain fatty alcohols with carbon backbones longer than 22 C (Juturu and Gormley, 2013). The effects reported by the Cuban researchers were ascribed to the unique composition of the fatty alcohols from Cuban-derived sugarcane wax. It was suggested that the cholesterol lowering effect of policosanol was due to inhibition of HMG-CoA reductase synthesis following hepatic conversion of fatty alcohols to their corresponding fatty acids (Menéndez et al., 2001).

Research groups outside Cuba have long failed to reproduce and validate the efficiency of policosanol in improving the lipoprotein profile (as reviewed by Marinangeli et al., 2010). However, a recent meta-analysis including 13 Cuban and 9 non-Cuban studies confirmed the efficacy and safety of sugarcane policosanol on dyslipidemia (Gong et al., 2018). In addition, experimental studies with Cuban policosanol in rats (Cho et al., 2018b), as well as clinical studies in healthy Korean subjects (Kim et al., 2017; Cho et al., 2018a; Kim et al., 2018) showed reduced body fat (Kim et al., 2017; Cho et al., 2018a) and improved blood lipid profile (Kim et al., 2017; Cho et al., 2018a; Cho et al., 2018b; Kim et al., 2018). It has been reported that this was due to inhibition of cholesteryl ester transfer protein (Kim et al., 2017).

Sugarcane wax is not the only source of (very) long-chain fatty alcohols that have been tested on human health. Montserrat-de la Paz et al. (2014) and Fernández-Arche et al. (2009) studied the anti-inflammatory effects of long-chain fatty alcohols from evening primrose oil and pomace olive oil, respectively. They showed that long-chain alcohols from both pomace olive oil and primrose oil inhibited TNFα and nitric oxide production in LPS-stimulated murine (M1) macrophages in a dose-dependent manner through inhibition of inducible

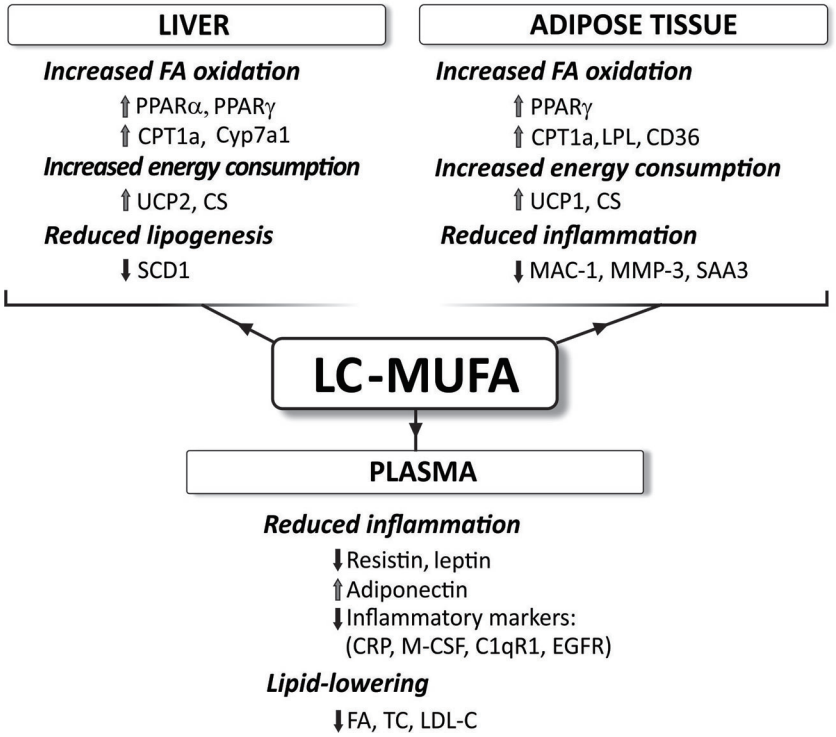

FIGURE 5 | Reported effects of concentrated LC-MUFA on gene expression related to fatty acid oxidation, energy consumption, lipogenesis, and inflammation in the liver and adipose tissue, as well as plasma levels of various compounds. C1qR1, complement component 1q receptor; CD36, fatty acid translocase; CPT1a, carnitine palmitoyltransferase 1a; CRP, C-reactive protein; CS, citrate synthase; CyP7a1, cholesterol 7 alpha-hydroxylase (cytochrome P450 7A1); EFGR, epidermal growth factor receptor; FA, free fatty acids; LDL-C, low-density lipoprotein cholesterol; LPL, lipoprotein lipase; MAC-1, macrophage-1 antigen; M-CSF, macrophage colony-stimulating factor; MMP-3, matrix metalloproteinase-3; PPAR, peroxisome proliferator-activated receptor; SAA3, serum amyloid A3; SCD-1, stearoyl-CoA desaturase-1; TC, total cholesterol; UCP, uncoupling protein (summarized from Yang et al., 2011d; Yang et al., 2013; Yang et al., 2015; Yang et al., 2016a; Yang et al., 2017).

nitric oxide synthase (iNOS). Pomace olive oil also decreased the production of the pro-inflammatory mediators prostaglandin E2 (PGE_2), in murine macrophages, and thromboxane B2 (TXB_2) in rat peritoneal neutrophils. Reduced release of these eicosanoids was due to inhibition of secretory phospholipase A2 ($sPLA_2$) (Fernández-Arche et al., 2009). The long-chain fatty alcohols from evening primrose oil had no effect on PGE_2 formation, but did cause a dose-dependent inhibition of the secretion of $sPLA_2$, TXB_2, and IL-1β in LPS-stimulated (M1) macrophages (Montserrat-de la Paz et al., 2014). The fatty alcohols from evening primrose oil also reduced the gene expression of cyclooxygenase-2, the enzyme needed for the production of eicosanoids, in a dose dependent manner (Montserrat-de la Paz et al., 2014).

Tetracosanol from sugarcane wax was shown to improve glycemic control *via* activation of insulin receptor kinase and translocation of GLUT 4 from the cytosol to the plasma membrane (Hsu et al., 2015). Recently, the health-promoting effects of policosanol and octacosanol have received new interest. Guo et al. (2017) showed that octacosanol improves the health status in a mouse model of colitis by reducing pathological damage in colonic tissue and inhibiting the gene and protein expression levels of TNFα, IL-1β, IL-6, and iNOS in the colon. Octacosanol also reduced the gene and protein expression of these pro-inflammatory cytokines in LPS-stimulated (M1) macrophages. Sharma et al. (2019) reported that policosanol and octacosanol supplementation reduced body fat gain, decreased insulin resistance, and reduced hepatic lipid content in high-fat diet-induced obese mice. This was associated with increased thermogenesis in brown adipose tissue due to GPR120 activation, as well as decreased expression of genes involved in lipogenesis and cholesterol uptake in the liver and reduced inflammation in white adipose tissue. Classical studies in rats have shown that fatty alcohols may be oxidized to their corresponding fatty acids (Stetten and Schoenheimer, 1940; Blomstrand and Rumpf, 1954) in the endoplasmic reticulum during hepatic metabolism and subsequent chain shortening in the peroxisomes (Hargrove et al., 2004). The effects of policosanol and octacosanol supplementation found by Sharma et al. (2019) were suggested to be due to the conversion of these fatty alcohols to their corresponding fatty acids.

Policosanols occur in different natural products (Shen et al., 2019; Weerawatanakorn et al., 2019). The fatty alcohols eicosenol (20:1n-9) and docosenol (22:1n-11) found in the wax esters in calanus oil are shorter in chain-length, compared to the different fatty alcohols in policosanols. It is therefore difficult to

extrapolate the health effects of the policosanols mentioned above to those in oil from *Cf*. However, Hsu et al. (2015) found that chain length of the policosanols did not affect their impact on glycemic control. Thus, it might be that the fatty alcohols found in calanus oil have similar effects as the policosanols.

Previous studies in our lab with calanus oil have demonstrated incorporation of the mono-unsaturated fatty acids, 20:1n-9 (in white adipose tissue) and 22:1n-11 (in liver) of mice fed a high-fat diet supplemented with the oil (Pedersen et al., 2014a). This could reflect the content of LC-MUFA in the oil, but also the *in vivo* oxidation of the corresponding fatty alcohols. This leads to the suggestion that the calanus oil-induced health effects may not be entirely due to the fatty acids, but indirectly also to the fatty alcohols in the oil. Calanus oil differs from other marine oils in terms of its content of fatty alcohols, and conversion of these alcohols to their corresponding monounsaturated fatty acids could boost the uptake of these specific fatty acids. The quantitative importance of this intriguing mechanism, as well as its metabolic implications, needs to be determined in new studies.

CONCLUSION

The oil from *Calanus finmarchicus* is a marine oil with a unique chemistry. Although relatively low in EPA and DHA, it contains high amounts of SDA and a number of monounsaturated fatty acids. The fatty acids are bound to long-chain fatty alcohols forming wax esters that constitute approximately 85% (w/w) of the oil. The various classes of fatty acids, as well as the fatty alcohols may have potential health benefits, since it is likely that fatty alcohols are oxidized to the corresponding fatty acids after absorption. This review has focused on the effect of the various components of calanus oil in relation to prevention of chronic low-grade inflammation, but more research is needed to determine the efficacy of the various components in this respect, or whether an anti-inflammatory effect of the oil is a result of the combined action of several components.

AUTHOR CONTRIBUTIONS

PS drafted the manuscript. PS, AP, K-EE, RO, and TL critically revised and edited the manuscript for clarity and content.

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Conflict of Interest: TL has a small position as scientific advisor in Calanus AS. AP is employed as product manager by Calanus AS.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Paper II

Obesity-induced alterations in the gut microbiome in female mice fed a high-fat diet are antagonized by dietary supplementation with a novel, wax ester–rich, marine oil

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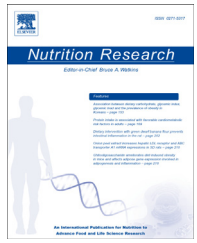

Obesity-induced alterations in the gut microbiome in female mice fed a high-fat diet are antagonized by dietary supplementation with a novel, wax ester–rich, marine oil

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ABSTRACT

Dietary supplementation with calanus oil, a novel wax ester–rich marine oil, has been shown to reduce adiposity in high-fat diet (HFD)–induced obese mice. Current evidence suggests that obesity and its comorbidities are intrinsically linked with unfavorable changes in the intestinal microbiome. Thus, in line with its antiobesity effect, we hypothesized that dietary supplementation with calanus oil should counteract the obesity-related deleterious changes in the gut microbiota. Seven-week-old female C57bl/6J mice received an HFD for 12 weeks to induce obesity followed by 8-week supplementation with 2% calanus oil. For comparative reasons, another group of mice was treated with exenatide, an antiobesogenic glucagon-like peptide-1 receptor agonist. Mice fed normal chow diet or nonsupplemented HFD for 20 weeks served as lean and obese controls, respectively. 16S rRNA gene sequencing was performed on fecal samples from the colon. HFD increased the abundance of the *Lactococcus* and *Leuconostoc* genera relative to normal chow diet, whereas abundances of *Allobaculum* and *Oscillospira* were decreased. Supplementation with calanus oil led to an apparent overrepresentation of *Lactobacillus* and *Streptococcus* and underrepresentation of *Bilophila*. Exenatide prevented the HFD-induced increase in *Lactococcus* and caused a decrease in the abundance of *Streptococcus* compared to the HFD group. Thus, HFD altered the gut microbiota composition in an unhealthy direction by increasing the abundance of proinflammatory genera while reducing those considered health-promoting. These obesity-induced changes were antagonized by both calanus oil and exenatide.

Abbreviations: Cal, calanus oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Ex, exenatide; GLP-1, glucagon-like peptide-1; HFD, high-fat diet; HFD + Cal, HFD supplemented with 2% (w/w) Calanus Oil; HFD + Ex, HFD plus treatment with exenatide; NCD, normal chow diet; PUFA, polyunsaturated fatty acids; SDA, stearidonic acid; WAT, white adipose tissue.

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1. Introduction

The role of gut microbiota in human health has received significant interest in recent years. Alterations in the composition of the gut microbiome have been associated with obesity and metabolic disorders, such as insulin resistance and type 2 diabetes mellitus, thereby increasing the risk for cardiometabolic disease [1-5]. The causal relationship between alterations in the gut microbiota and disease development is, however, still unclear, and it is not fully understood if changes in the microbial composition occur before or after disease onset [6]. However, it is known that diet is an important driver behind changes in the gut microbiota [7-9].

Humans have low ability to synthesize the long-chain omega-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA). It is therefore necessary to obtain these essential fatty acids via the diet. Studies describing the impact of EPA and DHA on the gut microbiota in humans are relatively sparse. Rajkumar et al. [10] were the first to study the impact of EPA and DHA on human gut microbiota but failed to detect any major effects on its composition. Later studies providing omega-3 PUFA supplements to both healthy and obese individuals revealed similar changes in both groups, such as a decrease in *Faecalibacterium*, often associated with an increase in the Bacteroidetes and butyrate-producing bacteria of the Lachnospiraceae family [11-13]. Animal studies have also reported alterations in gut microbiota following dietary supplementation with marine oils, and interestingly, these alterations in the microbiome were associated with reductions in body weight gain and white adipose tissue (WAT) inflammation [14] as well as a reduced adiposity index [15].

Calanus oil is a novel marine oil extracted from the copepod *Calanus finmarchicus*. The oil consists mainly of wax esters, a lipid class where fatty acids are esterified to long-chain fatty alcohols [16]. This novel marine oil is, compared to other marine oils, relatively low in EPA and DHA (6% and 4%, respectively) but relatively rich in stearidonic acid (18:4n-3, SDA, 7%), the precursor of EPA and DHA [17,18]. In addition, about 11% of the fatty acids present in the oil are (long-chain) monounsaturated fatty acids. The main fatty alcohols are represented by the equivalents of the dominant monounsaturated fatty acids, namely, eicosenol (20:1n-9) and docosenol (22:1n-11) [18].

Digestion and utilization of wax esters are poorly understood [19], although it is evident that humans are able, to some extent, to hydrolyze waxes and absorb the liberated fatty acids and alcohols [20]. Furthermore, previous studies in mice have shown that the wax esters are hydrolyzed and absorbed in the intestine and that the wax ester–derived fatty acids are incorporated in liver and WAT [18]. Dietary lipids, such as triacylglycerol and phospholipids, are quickly digested and absorbed in the upper parts of the gastrointestinal tract and will normally not reach the lower part [21]. Wax esters are hydrolyzed by a bile salt–dependent pancreatic carboxyl esterase [20]. They are relatively hydrophobic and therefore more difficult to emulsify, and as a result, wax esters may exhibit a longer retention time [22,23], allowing release of the liberated fatty acid and fatty alcohols in the distal part of the intestine.

The mouse model of diet-induced obesity shares many of the same characteristics as human obesity [24] and has become an important tool for understanding the pathological mechanisms involved in obesity-related diseases, such as insulin resistance, oxidative stress, and liver steatosis [25]. In particular, the development of a low-grade inflammatory state in obese adipose tissue and altered adipose tissue metabolism with increased release of fatty acids are believed to play a central role in the development of obesity-related pathology.

Previous studies reported that dietary supplementation with a small amount (2%) of oil from *C finmarchicus* significantly reduced intra-abdominal and ectopic fat deposition in male mice during high-fat feeding [26,27]. Because obesity is characterized by unfavorable alterations in the composition and function of the gut microbiome [4], we hypothesized that reduced obesity following intake of calanus oil could prevent or antagonize such alterations of the bacterial composition in the gut. To test this hypothesis, normal mice were made obese during a 12-week period on high-fat diet (HFD). This was followed by 8 weeks of feeding on HFD with or without 2% calanus oil. For comparative reasons, we also included a group of HFD mice that were treated with the antidiabetic compound exenatide. This glucagon-like peptide-1 (GLP-1) receptor agonist also reduces fat deposition in mice during high-fat feeding [28].

2. Methods and materials

2.1. Study design and animals

All animal experiments were approved by the local authority of the National Animal Research Authority in Norway (FOTS id 8430). All mice were treated according to the guidelines on accommodation and care of animals formulated by the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes. The animals were housed at 21°C, 3 animals per cage, under a reversed light/dark cycle (12-hour dark/12-hour light). The animals had ad libitum access to food and drinking water, and body weight and food intake were recorded once a week.

In this study, 4 groups of 5- to 6-week-old C57Bl/6J female mice (Charles River, Sulzfeld, Germany) were studied (5 mice per group). The first group served as the lean control group and was fed a normal chow diet (NCD) containing 10% of energy from fat (NCD, no. 58Y2, Test Diet; IPS Limited, Richmond, Indiana, USA) for 20 weeks. The other groups were diet-induced obese by feeding a lard-based high-fat diet containing 46% of energy from fat (HFD, no. 58V8, Test Diet; IPS Limited). The second group received the HFD for 20 weeks. The third and fourth groups received the HFD for 12 weeks followed by 8 weeks of treatment. The third group received HFD supplemented with 2% (wt/wt) commercial Calanus Oil, supplied by Calanus AS, Tromsø, Norway (HFD + Cal). The addition of the oil was compensated for by removal of 2 g lard/100 g diet, making the total fat and energy content of the HFD and the HFD + Cal similar. See Table 1 for a list of ingredients of the different diets and Table 2 for a list of fatty acid composition of Calanus Oil, the HFD, and the HFD + Cal. The nutritional profiles of the diets are shown in the supplementary data. The fourth group was fed HFD and treated with exenatide, 10 μg/kg/d, via miniosmotic pumps implanted subcutaneously at the back of the animals (HFD + Ex). After

Table 1 – Ingredient composition in g/kg of the 3 experimental diets

Ingredients	NCD	HFD	HFD + Calanus
Sucrose	331.29	200.92	200.92
Dextrin	298.56	84.83	84.83
Casein, vitamin tested	189.56	233.06	233.06
Powdered cellulose	47.39	58.27	58.27
Maltodextrin	33.17	116.53	116.53
Soybean oil	23.7	29.13	29.13
Lard	18.96	206.84	186.84
Calanus Oil-841	0	0	20
Potassium citrate, tribasic monohydrate	15.64	19.23	19.23
Calcium phosphate	12.32	15.15	15.15
DIO mineral mix	9.48	11.65	11.65
AIN-76A vitamin mix	9.48	11.65	11.65
Calcium carbonate	5.21	6.41	6.41
L-Cystine	2.84	3.5	3.5
Choline bitartrate	1.9	2.33	2.33
FD&C yellow no. 5	0.5	0	0
FD&C red 40 lake	0	0.5	0
Green dye	0	0	0.5
Total g/kg	1000	1000	1000
Total energy in kcal/g (kJ/g)	3.76 (15.73)	4.6 (19.25)	4.6 (19.25)

NCD, normal control diet (TestDiet 58Y2 with 10% energy from fat); HFD, high-fat diet (TestDiet 58V8 with 45% energy from fat); HFD + Cal, high-fat diet with 2% Calanus Oil (TestDiet 58V8 with 2% Calanus Oil). See supplementary materials for details.

implantation of the miniosmotic pumps, the mice were single housed to avoid animal interaction, which could damage the surgical wound. Mice that did not receive pumps were also single housed to control for possible effects of single housing.

Table 2 – Fatty acid composition (mg/g lipid) of Calanus Oil and experimental diets

Fatty acids	Calanus Oil	HFD	HFD + Cal
14:0	64.42	10.41	11.95
16:0	45.05	173.65	149.83
18:0	2.42	106.63	92.16
20:0	0.40	1.70	1.37
16:1*n-7*	17.17	10.95	9.98
18:1*n-7*	1.53	15.84	13.52
18:1*n-9*	15.54	243.82	208.42
20:1*n-9*	24.01	4.74	5.64
20:1*n-11*	3.90	ND	ND
22:1*n-9*	2.63	ND	ND
22:1*n-11*	43.33	ND	2.20
24:1*n-9*	2.81	ND	ND
18:2*n-6*	6.64	133.04	116.06
18:3*n-3*	13.72	12.49	11.67
18:4*n-3*	69.58	ND	4.54
20:2*n-6*	0.71	3.15	2.69
20:4*n-6*	1.39	0.48	1.15
20:5*n-3*	54.73	ND	3.35
22:5*n-3*	2.96	ND	ND
22:6*n-3*	39.35	ND	2.81

Modified from Pedersen et al. [18]. ND, not detected.

At the end of the feeding experiment, the mice were killed with an overdose of pentobarbital (100 mg/kg, 300 μL intraperitoneally). Organs were carefully dissected out and stored at –80 °C until further analysis. Feces samples were collected from the colon for microbiota analysis, after which the gastrointestinal tract was rinsed with saline before freezing.

2.2. *Fatty acid analysis of the colon wall and abdominal fat*

Fatty acids were methylated by dissolving 60 mg tissue sample in 2 mol/L HCl in methanol with 0.05% BHT and heating for 2 hours at 100 °C, and the fatty acid composition was determined by gas chromatography using an Agilent 6890N (Agilent Technologies, Santa Clara, CA, USA) gas chromatograph with a Varian CP7419 capillary column (Varian Inc, Palo Alto, CA, USA). The content of the individual fatty acids in the samples was expressed in percent of the total fatty acid content.

2.3. *Quantitative real-time polymerase chain reaction*

The expression of mRNA of genes related to inflammation and fat metabolism in the colon and omental WAT was determined using quantitative reversed real-time polymerase chain reaction (PCR). Samples of the intestinal wall (colon, n = 6-8 per group) were immersed in RNA later (Qiagen, Hilden, Germany), and WAT samples (n = 6-8 per group) were immersed in Allprotect Tissue Reagent (Qiagen, Hilden, Germany) and thawed overnight at 4°C. Thirty to 40 mg colon tissue and 50-100 mg WAT tissue were used for RNA extraction according to the Rneasy Lipid Tissue kit Protocol (Qiagen). RNA concentrations were measured spectrophotometrically (Nanodrop 2000, Thermo Fisher Scientific, Waltham, MA, USA) and stored at –80 °C before use for cDNA. cDNA was subsequently made from 2 μg total RNA according to High Capacity cDNA reverse transcriptase kit (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was stored at –20 °C until real-time PCR (quantitative PCR) was performed in a Roche LightCycler 96 using a 1:4 dilution of the cDNA and the fast-start essential DNA green master (Roche, Basel, Switzerland). Five housekeeping genes were analyzed to normalize the expression of the target genes to the geometric mean of the 2 best housekeeping genes, which were selected on the basis of the average expression stability values determined with geNorm. For quantification of the gene expression in the colon, the housekeeping genes *sdha* (succinate dehydrogenase complex subunit A) and *hprt* (hypoxanthine-guanine phosphoribosyl transferase) were used, and for the WAT, *sdha* and *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) were used. Forward and reverse primers of the target genes analyzed in the colon and omental fat are shown in Table 3.

2.4. *Bacterial DNA isolation*

Total microbial DNA was isolated from feces samples with a QIAamp PowerFecal DNA kit (Qiagen) according to the manufacturer's protocol, with the following exceptions. The disintegration of the cells was done with a FastPrep device at a vortexing speed of 6.5 m/s for 30 seconds followed by

Table 3 – Forward and reverse primers of the target genes analyzed in the intestinal (colon) wall and omental fat

Gene	Accession number	Primer	Melting temperature (°C)	Product size
SDHA	NM_023281	F: TGT TCA GTT CCA CCC CAC A R: CAC GAC ACC CTT CTG TGA TG	59.0 59.0	62 bp
GAPDH	NM_001289726.1	F: TCA CCA CCA TGG AGA AGG C R: GCT AAG CAG TTG GTG GTG CA	59.3 61.2	169 bp
HPRT	NM_013556.2	F: TCC TCC TCA GAC CGC TTT T R: CCT GGT TCA TCA TCG CTA ATC	58.2 56.6	90 bp
NFkB	NM_008689.2	F: ATG GCA GAC GAT GAT CCC TAC R: CGG AAT CGA AAT CCC CTC TGT T	59.4 60.4	167 bp
GPR 120	NM_181748.2	F: GTG CCG GGA CTG GTC ATT GTG R: TTG TTG GGA CAC TCG GAT CTG G	63.8 62.3	123 bp
EMR1	NM001355722.1	F: TTG TAC GTG CAA CTC AGG ACT R: GAT CCC AGA GTG TTGATG CAA	59.6 58.3	144 bp
CD36	NM_001159558.1	F: TTG TAC CTA TAC TGT GGC TAA ATG AGA R: CTT GTG TTT TGA ACA TTT CTG CTT	59.7 57.4	72 bp
MCP1	NM_011333.3	F: TTA AAA ACC TGG ATC GGA ACC R: GCA TTA GCT TCA GAT TTA CGG)	56.2 55.1	121 bp
TLR4	NM_ 021297.3	F: TTC TTC TCC TGC CTG ACA CC R: CTT TGC TGA GTT TCT GAT CCA T	59.3 56.7	94 bp
Adiponectin	NM_009605.5	F: CCT GGC CAC AAT GGC ACA CCA R: GTG ACG CGG GTC TCC AGC C	65.8 65.5	233 bp
PPARα	NM_011144.6	F: ACG ATG CTG TCC TCC TTG ATG R: GTG TGA TAA AGC CAT TGC CGT	60.1 59.5	67 bp
PPARδ	NM_011145.3	F: GCT GCT GCA GAA GAT GGC A R: CAC TGC ATC ATC TGG GCA TG	61.0 59.3	63 bp
PPARγ	NM_001127330.2	F: CCA TTC TGG CCC ACC AAC R: AAT GCG AGT GGT CTT CCA TCA	58.3 59.7	67 bp
FIAF	NM_020581.2	F: GCC ACC AAT GTT TCC CCC AAT G R: TAC CAA ACC ACC AGC CAC CAG AGA	62.6 65.7	118 bp
IL-1β	NM_008361.4	F: TGT AAT GAA AGA CGG CAC ACC R: TCT TCT TTG GGT ATT GCT TGG	58.9 56.1	68 bp
MUC2	NM_023566.4	F: ATG CCC ACC TCC TCA AAG AC R: GTA GTT TCC GTT GGA ACA GTG AA	59.7 59.1	101 bp
GLP2R	NM_ 175 681.3	F: TCA TCT CCC TCT TCT TGG CTC TTA C R: TCT GAC AGA TAT GAC ATC CAT CCAC	61.6 60.0	196 bp
IL-18	NM_008360.2	F: CAT GTA CAA AGA CAG TGA AGT AAG AGG R: TTT CAG GTG GAT CCA TTT CC	59.9 55.3	122 bp
ZO-1	NM_009386.2	F: GAG CGG GCT ACC TTA CTG AAC R: GTC ATC TCT TTC CGA GGC ATT AG	60.5 59.0	75 bp
IFNγ	NM_008337.4	F: TTG GCT TTG CAG CTC TTC CT R: TGA CTG TGC CGT GGC AGT A	60.2 61.5	58 bp
Occludin	NM_008756.2	F: TTG AAA GTC CAC CTC CTT ACA GA R: CCG GAT AAA AAG AGT ACG CTG G	59.0 59.1	129 bp

incubation at 70 °C for 5 minutes. The vortexing and incubation were repeated once. The elution was done with 100 μL of elution buffer and stored at –20 °C until used.

2.5. *16S rDNA amplification*

Twenty nanograms of DNA was used to prepare PCR amplicons of the V4-V5 region of 16S rRNA according to Fliegerova et al. [29]. The mixture contained OneTaq DNA Polymerase (New England Biolabs, Ipswich, Massachusetts, USA), and the thermal profile consisted of initial denaturation for 5 minutes at 95 °C followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at 57 °C, and 30 seconds at 72 °C and a final elongation for 5 minutes at 72 °C. The PCR amplicons were checked by electrophoresis in 1.5% agarose (25 minutes at 90 V), purified with a QIAquick PCR Purification Kit (QIAGEN) according to the protocol, and quantified by Nanodrop OneC.

2.6. *Next-generation sequencing*

The obtained PCR products were used to prepare amplicon libraries for diversity analyses by a next-generation sequencing approach on a Personal Genome Machine (Life Technologies, Carlsbad, California, USA) according to Milani et al. [30]. Two hundred nanograms of DNA from each sample was used to prepare sequencing libraries with a NEBNext Fast DNA Library Prep Set kit (New England Biolabs) according to the manufacturer's protocol. Ion Xpress Barcode adapters (Thermo Fisher Scientific) were used to label each sample. The obtained libraries were used to prepare a sequencing template by using an Ion PGMTM Hi-QTM View OT2 Kit (Thermo Fisher Scientific). The template was then sequenced on an Ion 316TM Chip Kit v2 (Thermo Fisher Scientific) by using an Ion PGMTM Hi-QTM View Sequencing kit (Thermo Fisher Scientific). Two independent sequencing runs were performed.

2.7. Next-generation sequencing data analyses

The sequences were obtained in the form of the FASTQ format and further processed by the QIIME2 analyses pipeline [31]. The sequences were demultiplexed and dereplicated using the versatile and open-source tool VSEARCH [32]. The bioinformatics software PICRUSt v 1.1.3 was applied for both the taxonomical and functional analyses [33]. The sequences were clustered and identified by performing closed-reference operational taxonomic unit (OTU) picking selecting a cutoff at 90% sequence identity against the Greengene reference OTUs (gg_13_5_otus). The resulting OTU table was normalized using the *normalize_by_copy_number.py* script. Functional trait abundance was predicted using the *predict_metagenomes.py* script. The predictions were collapsed into Kyoto Encyclopedia of Genes and Genomes pathways level 3 using the *categorize_by_function.py* script, and the resulting abundance table was imported in the bioinformatics software STAMP for statistical analysis [34].

Diversity index analysis and unweighted UniFrac distance metrics analysis [35] were carried out by exporting the QIIME2 biom file to MicrobiomeAnalyst. The Web site microbiomeanalyst.ca was used for visualization of the α diversity index and the heat map. The absolute and relative abundance tables were exported and used in STAMP for statistical analysis of the taxonomy data, in GraphPad prism 8.2.1 for the (stacked) bar graphs, and in MEGAN Community Edition (version 6.15.2) for visualization of the principal component analysis.

2.8. Statistical analyses

Data are presented as the means with their standard errors. Statistical analysis of the functional and taxonomic data was done using STAMP [34]. Noncorrected 2-sided Student *t* test between the HFD group and the other three groups was done to check for significant differences vs the HFD group (n = 5 for each group). Mean differences were considered significant at $P < .05$. Statistical analysis on the gene sequencing was done in GraphPad prism 8.2.1. Differences between treatment groups were assessed by 1-way analysis of variance followed by Dunnet post hoc test where the groups were compared to the HFD group (n = 6-8 for each group). Sample sizes were determined based on availability of the samples because this study was ran in parallel to another study using the same mice [36].

3. Results

3.1. Body weight gain and fatty acid composition of the diets, and abdominal fat and colon wall

The present microbiota data were obtained on a subgroup of mice (n = 5) used in a recent publication by Jansen et al. [36]. Details about body weight development, weight of adipose tissue, and food intake are given in that publication. Here it is just pointed out that supplementation of the HFD with oil from *C finmarchicus* for the last 8 weeks did not affect body weight gain (Fig. 1), although it did result in a lower adiposity index (total weight of intra-abdominal fat depots) relative to the nonsupplemented HFD group. Treatment with exenatide, however, led to both lower weight gain (Fig. 1) and lower adiposity index. Of note, both treatments recovered the capacity of the heart to oxidize glucose, which was otherwise impaired in response to high-fat feeding [36], demonstrating that both the wax ester–rich oil and exenatide recovered metabolic flexibility of hearts from diet-induced obese mice. There were no differences in food intake between the diet groups during the treatment period [36]. The inclusion of 2% calanus oil in the HFD resulted in clearly detectable amounts of SDA, EPA, and DHA in this diet (Table 2). Analysis of the fatty acid composition of abdominal fat showed a statistically significant increase in the content of n-3 PUFA (SDA, DHA, and EPA), whereas the content of arachidonic acid (n-6 PUFA) was significantly reduced (Table 4). In the colon wall, the content of SDA was significantly increased ($P < .05$) in the HFD + Cal group relative to that of the HFD group (0.89% ± 0.23% vs 0.63% ± 0.07%), but for the other PUFA, the variability was too high to reach firm conclusions (data not shown).

3.2. Microbiota composition of feces samples from colon

The average sequence count over all 20 samples was 6761 sequence reads per sample. The average species diversity, or α diversity, within the 4 diet groups was measured using the Shannon Phylogenetic Diversity index on the OTU level (Fig. 2). No statistically significant differences were observed between the groups ($P = .0596$, Kruskal-Wallis statistics: 7.4229).

The β diversity, the difference between the diet groups, is presented through a principal coordinate analysis based on unweighted UniFrac distances (Fig. 3). The first and second principal coordinates are given. Axis 1 explains 43.7% of the variation, and axis 2 explains 22.6%. Distinct clusters separate the NCD and HFD + Ex groups. The HFD and HFD + Cal groups overlap, with the HFD + Cal group having a larger cluster than the HFD group.

Fig. 4A illustrates the relative abundance of the different bacterial phyla for each diet group. Firmicutes was the most abundant phylum in all 4 groups, followed by Bacteroidetes. There were no apparent differences in the relative abundance on the phylum level. But there were significant differences on the genus level as highlighted in Fig. 4B. It is obvious that the HFD and HFD + Cal groups share a similar bacterial profile, whereas the profiles of the HFD + Ex and NCD groups are different compared to HFD. To illustrate the relative abundance of the various genera in more detail, we also generated bar graphs ± standard error of the mean in Fig. 5. The most abundant genera in the feces samples were *Allobaculum*, *Lactobacillus*, *Lactococcus*, *Turicibacter*, and *Parabacteroides*. On first sight, it seemed that the main effect of HFD is a marked decline in *Allobaculum* and the presence of *Lactococcus* relative to the NCD group. Dietary supplementation with calanus oil did not seem to influence these HFD-induced changes in the microbiota composition, except for an apparently further decline in *Allobaculum* and an increased abundance of *Lactobacillus*. Treatment with exenatide reduced the high abundance in *Lactococcus* present in the HFD group. *Turicibacter* was abundant in all diet groups but mostly in the NCD and HFD + Ex group, whereas the presence of *Parabacteroides* seemed to be independent of diet.

A noncorrected 2-sided Student *t* test (comparing HFD vs NCD, HFD vs HFD + Cal, and HFD vs HFD + Ex) revealed that,

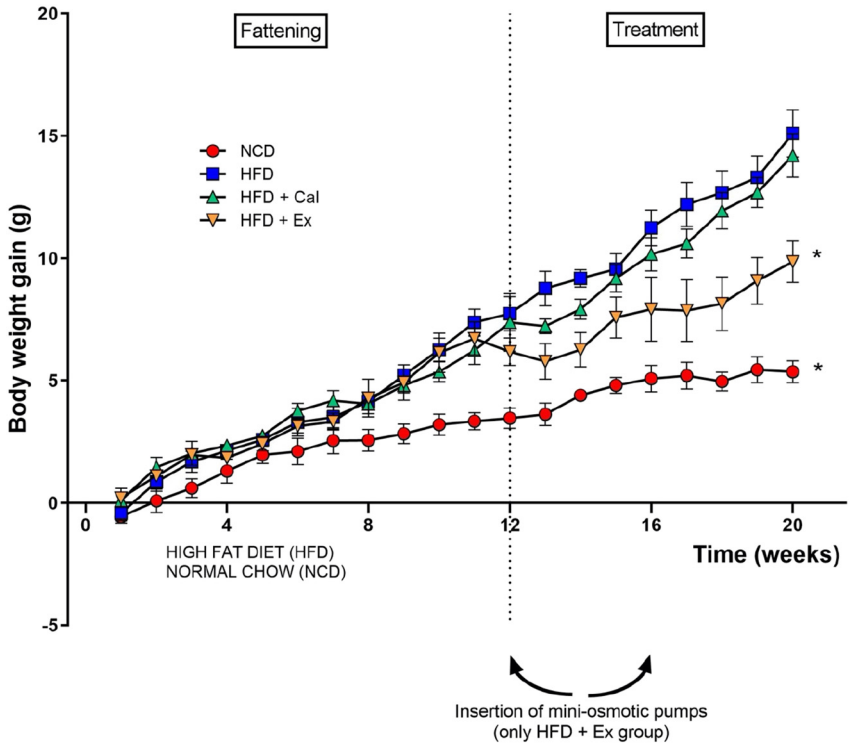

Fig. 1 – Body weight gain for the four different diet groups. Data are shown as means ± SEM. After 8 weeks of treatment, the weight gain of NCD and HFD + Ex groups was significantly different from HFD ($P < .05$).

indeed, the decrease in *Allobaculum* and increase in *Lactococcus* in the HFD compared to the NCD are significantly different, as was the increase in *Leuconostoc* (Fig. 6A). There were no significant differences between the HFD and HFD + Cal group. The apparent decrease in *Lactococcus* in the exenatide-treated group compared to the HFD group was significantly different, as well as the decrease in *Streptococcus* (Fig. 6B).

It is currently unknown at what abundance threshold certain gut bacteria lead to an increased disease risk. A statistical difference might therefore not necessarily lead to pathology or health improvement. Therefore, we visualize in Fig. 7 the differences of the various genera for each individual fecal sample in more detail with a heat map. Difference in colored tiles represents the multiplication of abundance of the genera, per sample, compared to the average abundance of those genera of all 20 fecal samples.

Table 4 – Content of PUFA in % of total fatty acid content in intra-abdominal fat from the various groups of mice

	SDA	DHA	EPA	AA
NCD	0.78 ± 0.15	0.26 ± 0.17	ND	0.42 ± 0.12
HFD	0.65 ± 0.03	0.19 ± 0.03	ND	0.40 ± 0.04
HFD + Cal	1.09 ± 0.12[a]	0.74 ± 0.10[a]	0.19 ± 0.04[a]	0.30 ± 0.04
HFD + Ex	0.71 ± 0.09	0.23 ± 0.04	ND	0.44 ± 0.09

Results are mean ± SD (n = 5 in each group).
[a] $P < .05$ vs HFD.

Some genera showed large variation in abundance within the diet groups (*Bacteroides*, *Parabacteroides*, *Lactobacillus*, *Akkermansia*, *Adlercreutzia*, *Coprococus*, *Blautia*, *Turicibacter*, *Bilophila*), whereas the abundance was more stable for other genera (*Lactococcus*, *Lauconostoc*, *Allobaculum*, *Ruminococcus*, *Streptococcus*, *Dorea*, *Oscillospira*). Thus, in line with the relative abundance shown in Fig. 5, the heat map shows that *Lactococcus*, *Leuconostoc*, and *Streptococcus* were above average present in the HFD and HFD + Cal groups, whereas *Dorea* and *Ruminococcus* were below average present in these groups. *Allobaculum* and *Oscillospira* were below average present in the 3 groups feeding on an HFD. Moreover, feces samples from the majority of mice in the HFD + Cal group were characterized by overrepresentation of *Lactobacillus* and *Streptococcus* and underrepresentation of *Bilophila*. In the HFD + Ex group, overrepresentation of *Ruminococcus* was particularly evident.

3.3. *Metagenomic functional prediction*

The 16S rRNA gene was used as a marker gene to extrapolate the taxonomic findings into functional predictions. The taxonomic information from screening samples against the Greengenes reference database was used to extract information from the complete genome sequences of the identified species. Combining whole genome information together with abundance measures for each species allowed for the prediction and quantification of functional pathways. Statistical analysis of the Kyoto Encyclopedia of Genes and Genomes data identified in total 328 genes related to

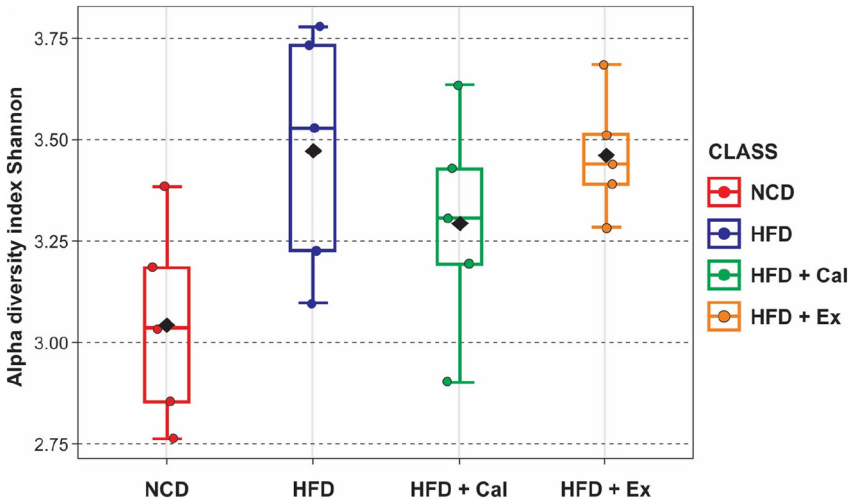

Fig. 2 – *α* diversity measured as Shannon Phylogenetic Index on OUT level for the 4 different diet groups. There were no statistically significant changes in the *α* diversity between the groups. (P = .0596, Kruskal-Wallis statistics: 7.4229).

functional categories (proteins) (Supplementary Table S1). Significant differences in the relative abundance of the predicted microbial genes (based on the noncorrected 2-sided Student t test) revealed the difference in the mean proportions of these genes between the HFD and NCD groups, the HFD and HFD + Cal groups, and the HFD and the HFD + Ex groups (Figs. 8A-C). As can be seen in Fig. 8A, 38 categories were differently expressed in the HFD vs NCD groups. Relevant for the current study was the overexpression of lipid and fatty acid biosynthesis proteins in the gut microbiota of the HFD-fed mice. Fig. 8B gives the 7 functional categories that were differently present in the colonic microbiota in the HFD and HFD + Cal groups. The novel marine oil upregulated 4 of the 7 categories in the high-fat–fed mice, which included proteins related to glycolysis, gluconeogenesis, and phosphate transferase systems (Fig. 8B). Ten categories where differently expressed in the microbiota of the HFD vs the HFD + Ex group (Fig. 8C). With respect to metabolic control, we note that proteins involved in glycine, serine, and threonine metabolism were overexpressed in the HFD + Ex group.

3.4. *Gene expression*

The gene expression of several markers for inflammation, fat storage regulation, and permeability of the intestinal wall

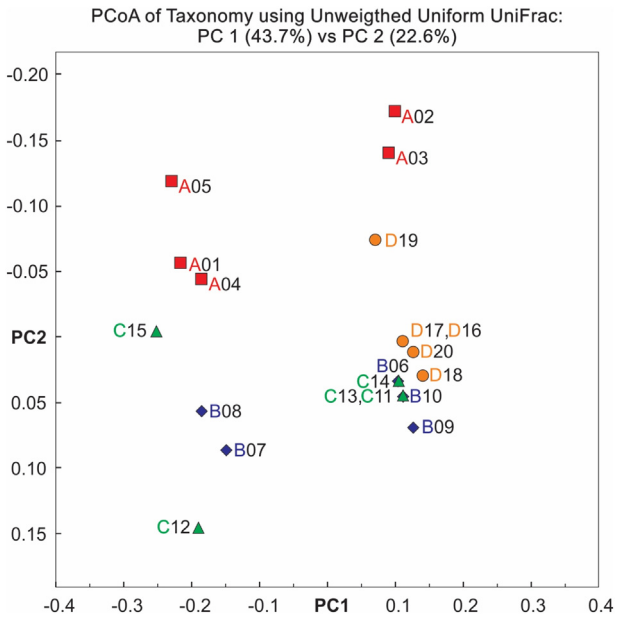

Fig. 3 – Principle coordinate analysis based on unweighted UniFrac in mice fed different diets. The symbols indicate values for individual mice. Percent variation explained by the principal coordinates PC 1 and PC 2 was 43.7% and 22.6%, respectively. Red squares, NCD; blue diamonds, HFD; green triangles, HFD + Cal; orange circles, HFD + Ex.

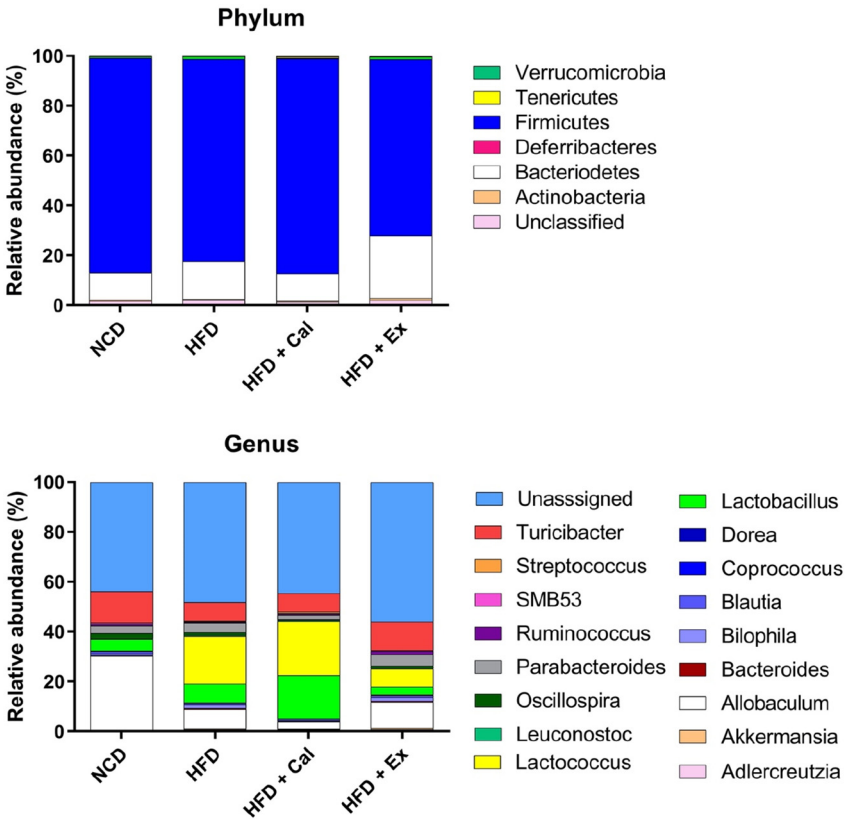

Fig. 4 – Relative abundance at the phyla (A) and genera levels (B) in the 4 diet groups.

were measured in the omental WAT (GPR120, IL-1β, IL-6, IL-10, TNF-α, CD36, EMR1a, adiponectin TLR4, MCP1, NF-κB, FIAF, PPARα, PPARβ, PPARγ, and GLP2 R) and the colon (GPR41, GPR43, GPR120, TNF-α, MUC2, GLP2 R, IL-1β, Il-10, IL-18, IFN-γ, FIAF, ZO-1, occluding, TLR4, and NF-κB). None of the analyzed genes were up- or downregulated depending on diet in the 2 tissues, with the exception of adiponectin (see also Supplementary Figs. S1 and S2).

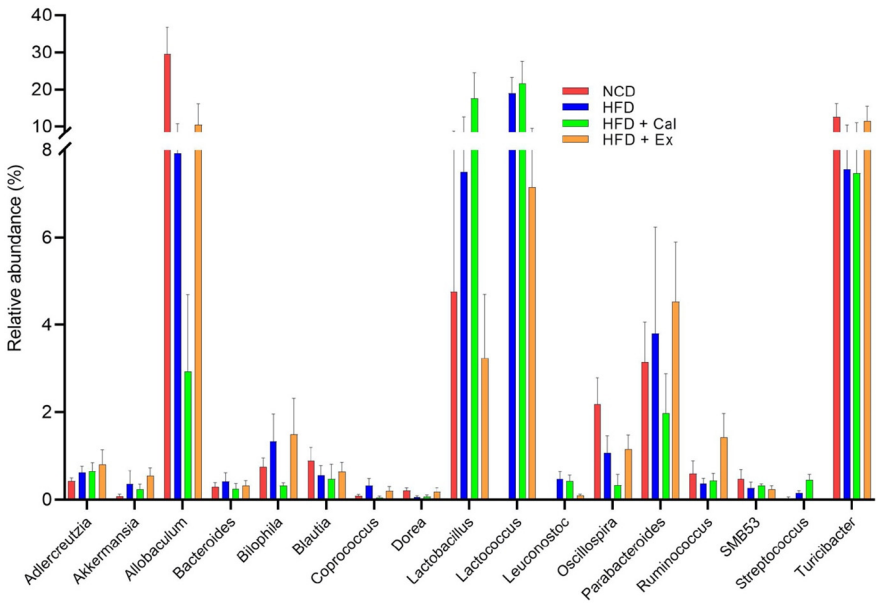

Fig. 5 – Relative abundance of the different genera present in colon feces samples from the 4 diet groups. Data shown as means ± SEM.

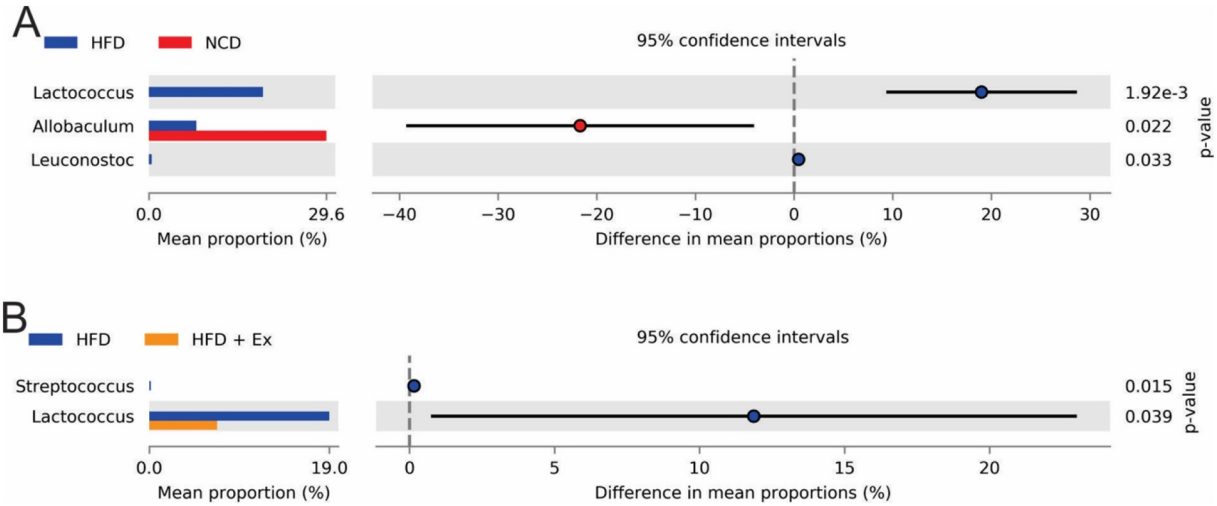

Fig. 6 – Comparison of mean proportion of bacterial genera. (A) HFD vs NCD groups. (B) HFD vs HFD + Ex groups. Only statistically significant differences ($P < .05$, noncorrected 2-sided Student t test) are shown.

4. Discussion

Diet has long been considered the most important driver behind changes in the intestinal microbiota [7-9]. In this study, we show that long-term feeding on a HFD led to an increase in the intestinal abundance of the bacterial genera *Lactococcus* and *Leuconostoc*, whereas the abundance of *Allobaculum* and *Oscillospira* was decreased. Supplementation of the HFD with 2% of wax ester–containing oil from *C*

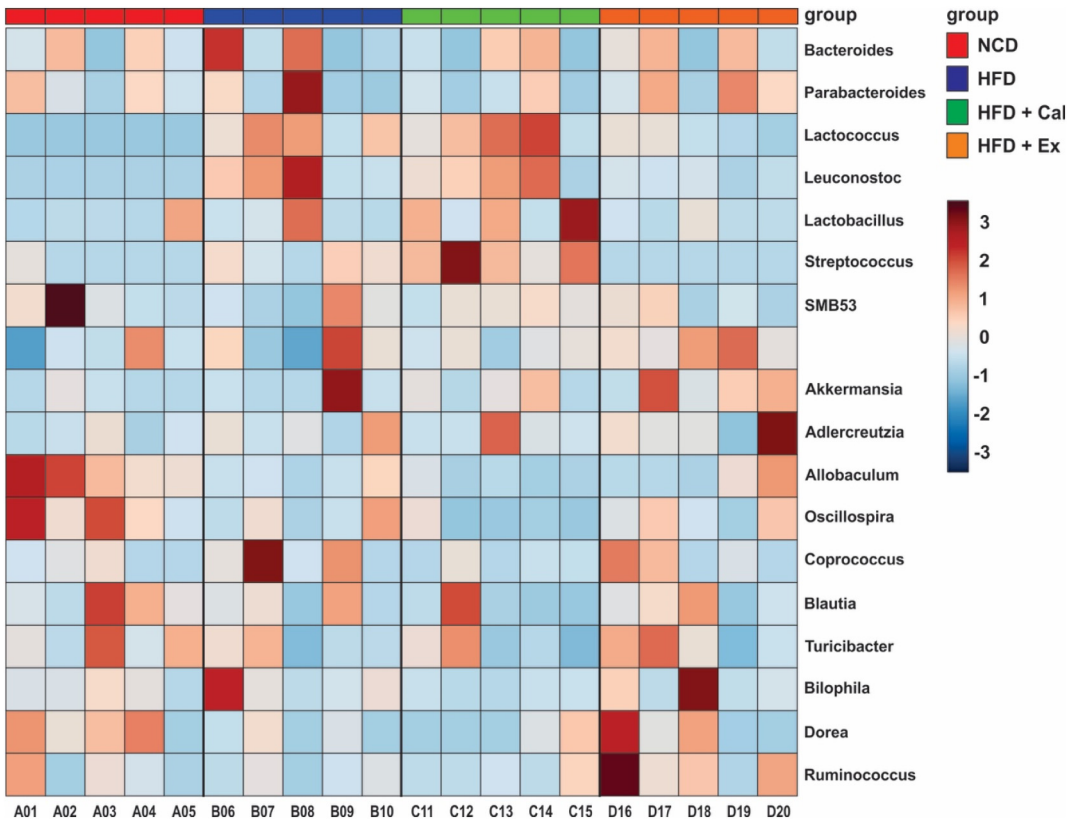

Fig. 7 – Heat map showing the representation of the different genera in each individual fecal sample compared to the average abundance of those genera. The multicolored bar at the top delineates the 4 diet groups, whereas the individual samples are indicated at the bottom of the heat map. The colored tiles indicate a positive (light to dark brown) or negative multiplication (light to dark blue) of the genus average across all individual samples.

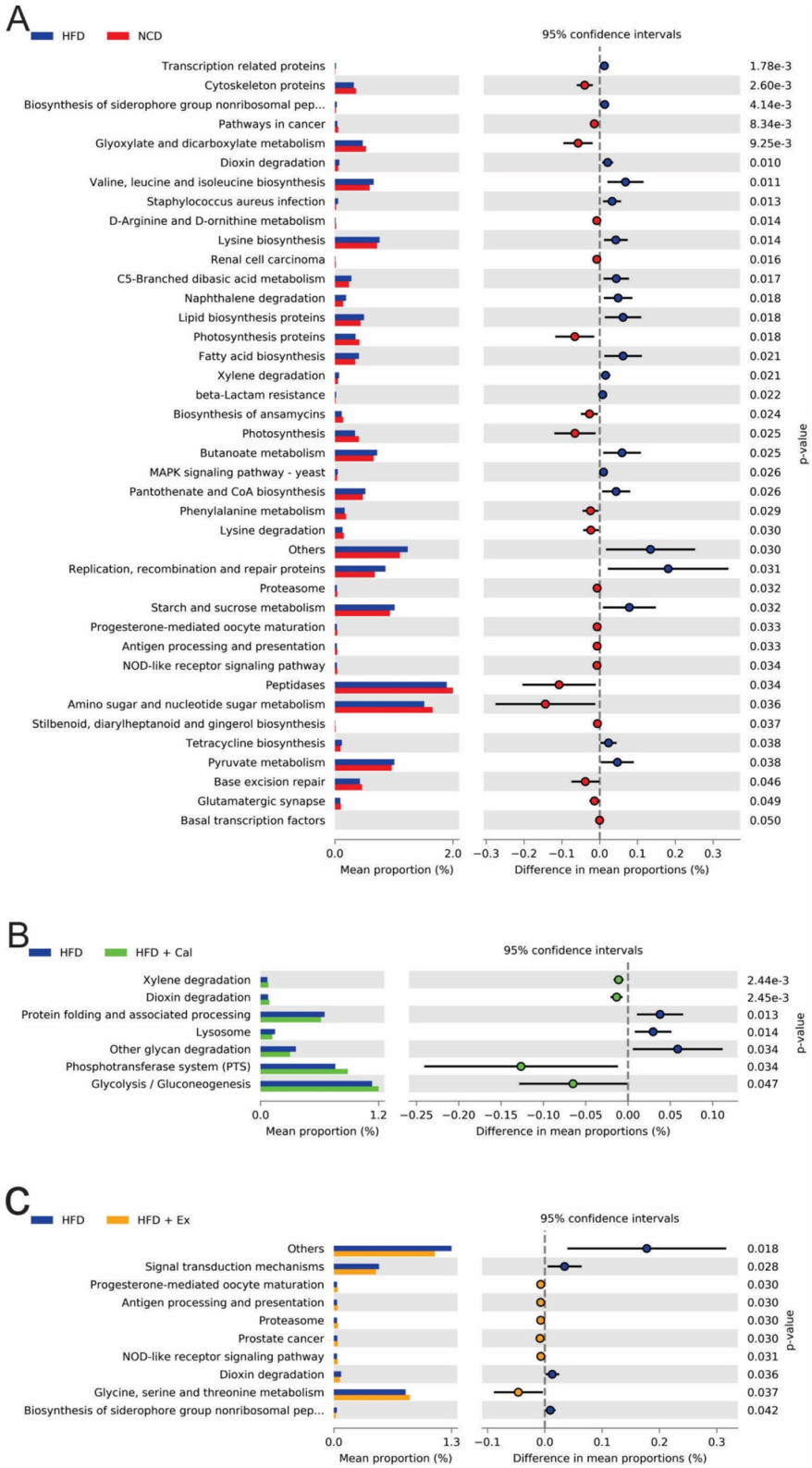

Fig. 8 – Comparison of mean proportion of predicted microbial genes present in colonic samples related to functional categories of (A) HFD vs NCD, (B) HFD vs HFD + Cal, and (C) HFD vs HFD + Ex. Only significant ($P < .05$) functional pathways are shown based on a noncorrected 2-sided Student t test. The colored circles represent the difference in mean proportions of the various functional categories with the 95% confidence interval, as calculated using bootstrap method.

finmarchicus did not alter this pattern significantly, although it led to an overrepresentation of *Lactobacillus* and *Streptococcus* and underrepresentation of *Bilophila*. Administration of the GLP-1 analog exenatide prevented the HFD-induced increase in *Lactococcus*, and in addition, it caused a decrease in the abundance of *Streptococcus*.

The high relative abundance of *Lactococcus* and the near depletion of *Allobaculum* in the HFD group are in agreement with previous animal studies [8,9]. *Lactococcus* is associated with proinflammatory markers [8,13,15], whereas *Allobaculum* is a short-chain fatty acid–producing bacterium which is considered health-promoting. Therefore, HFD used in the current study seems to change the microbiota composition in an unhealthy direction. This view was further supported by the finding that *Oscillospira*, a genus of importance for human health [37] and which has been reported to correlate negatively with body mass index and paracellular permeability in humans [38], was depleted in nearly all HFD mice.

Dietary fat has been shown to modulate gut microbiota [39], and to our knowledge, this is the first study investigating the effect of the oil from *C finmarchicus* on gut microbiota. As mentioned in the introduction, this marine oil contains more than 85% wax esters, consisting of saturated, monounsaturated, and polyunsaturated fatty acids bound to long-chain fatty alcohols. The general view is that the omega-3 fatty acids EPA and DHA represent the active substance in marine oil preparations, but the high content of SDA (7%) and fatty alcohols (about 350 mg/g), which is unique for this novel marine oil, could also be beneficial for human health, [40]. To which extent this fatty acid and fatty alcohols affect the composition of the gut microbiota is, to our knowledge, not known.

Supplementing HFD with 2% calanus oil was not able to restore the microbiota composition to that of the NCD group. Nevertheless, the relative abundance of *Lactobacillus* and *Streptococcus* was above average in the majority of the mice receiving the marine oil. This observation is in line with results by Caesar et al. [14], who reported increased abundance of *Lactobacillus* and *Streptococcus* when they replaced the lard in the high-fat diet with fish oil (Menhaden oil). Our results are also in line with findings by Mujico et al [41], who observed an increase in *Lactobacillus* following supplementation of HFD with EPA and DHA in female mice. Caesar et al. [14] reported reduced body weight gain and reduced inflammation due to an interaction between dietary lipids and the microbiota, which reduced toll-like receptor 4 (TLR4)–induced secretion of monocyte chemoattractant protein-1 (MCP-1/CCL2). Unlike the study by Caesar et al. (2015), we did not detect any differences between the groups with respect to WAT or colon gene expression of MCP-1, TLR4, and other inflammatory markers, which could be due to a relatively short treatment period with the marine calanus oil used in the present study. We observed an antiobesogenic effect of the wax ester–rich oil in terms of a reduced adiposity index. The mice did not, however, show a clear reduction in body weight development, as seen in previous experiments [26,27], and this could be due to a relatively short treatment period (8 vs 27 weeks). In addition, it should be noted that we used female mice in the current experiment and therefore cannot exclude gender differences with respect to the response of the marine oil supplementation. We did however observe an increased level of n-3 PUFA (and decline in n-6 PUFA) in intra-abdominal fat of mice receiving HFD supplemented with calanus oil, which is in line with previous results [18] and demonstrates very clearly that the wax ester in the oil is digested in the intestine is taken up, and thereby contributes to a healthier adipose tissue. In light of the role of omega-3 fatty acids as PPAR ligands [42] and the consequent activation of gene transcription, it was a bit surprising that the reduced adiposity index following calanus oil supplementation was not reflected in any significant change in PPAR expression in adipose tissue. One possibility could be that PPARα was upregulated primarily in metabolically active tissues, such as liver, heart, and skeletal muscle, leading to increased fatty acid oxidation and thereby draining of fatty acids from WAT.

The increased abundance of *Lactobacillus* might be of biological relevance because it is considered a health-promoting, short-chain fatty acid–producing probiotic strain that is often associated with weight loss, regulation of fat metabolism, and anti-inflammation (as reviewed in [13] and [43]). Most species of *Streptococcus* are considered pathogenic, but, for example, *Streptococcus thermophilus* is a probiotic. Unfortunately, our analysis was not capable of distinguishing between the different species of this genus, so the impact of the increased abundance of *Streptococcus* is not clear. Caesar et al. [14] also found a lower abundance of *Bilophila* in the fish oil–fed mice compared to the lard-fed mice. Interestingly, all 5 animals fed HFD + Cal in the current study also showed a below average abundance of *Bilophila*. This genus is shown to increase upon consumption of diets rich in saturated animal fats, and increased abundance of *Bilophila wadsworthia* is associated with inflammatory bowel disease [44]. To find out if the decreased adiposity index in response to dietary oil, as observed in the present study, was due to an enrichment of *Lactobacillus* and/or depletion of *Bilophila*, a larger study needs to be done.

In recent years, the effect of antidiabetic drugs on the gut microbiota has received more and more attention both in humans and in animal models. For example, it is known that the most used antidiabetic drug, metformin, as well as different α-glucosidase inhibitors (acarbose, miglitol, and voglibose), alters the composition of the gut microbiota [45,46]. A few studies examined the effect of liraglutide which, like exenatide, is a GLP-1 receptor agonist [47-49]. Treatment with liraglutide was associated with an increase in *Blautia* and *Turicibacter*, whereas the effect on *Allobaculum* and *Lactobacillus* was less clear and dependent on the animal model in question [47,48]. Wang et al. [47] found that liraglutide-induced weight loss was associated with, among others, *Lactobacillus*, *Turicibacter*, *Coprococcus*, and *Blautia*. The exenatide-treated mice in our study showed decreased body weight and fat mass compared to the HFD mice, and in line with Wang et al. [47], we observed a slight increase in the abundance of *Turicibacter* in this group. However, we did not observe any effect of exenatide on the other "weight reducing" bacteria. Interestingly, exenatide seemed to reduce the abundance of *Streptococcus* and *Lactococcus*, which are considered pathogenic, while increasing the abundance of *Ruminococcus* and *Dorea*. The latter 2 genera are generally related to insulin resistance and disturbed metabolic health [50,51]. In addition, De Filippis et al. [52] found a correlation between *Ruminococcus* and trimethylamine N-oxide levels, a microbiota-dependent metabolite derived

from trimethylamine-containing nutrients that are abundant in a Western diet and has been associated with obesity, insulin resistance, and increased thrombosis potential in animal and clinical studies [53]. Thus, the previously reported improvement in insulin sensitivity following exenatide treatment [28] could imply the effect of reduced abundance of *Streptococcus* and *Lactococcus* overrides that of the increase in *Ruminococcus* and *Dorea*.

It is currently unknown how GLP-1 receptor agonists induce alterations in the microbiota profile, but changes in pH and nutrient composition in the gut, as well as delay in gut transit time and gastric emptying rate, could be involved [47]. Another proposed mechanism is via the interplay with bile acids [51]. GLP-1 receptor agonists are suggested to induce weight loss via reduced food intake due to inhibition of appetite and reduced gastric emptying. In our study however, we could not detect any difference in food intake between the HFD-fed groups. We therefore suggest that weight loss in response to exenatide treatment could be due to a re-establishment of the abundance of *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Turicibacter*, *Dorea*, and *Ruminococcus* which characterized the lean NCD group.

Functional analysis of the gut microbiota revealed significant changes between the NCD- and the HFD-fed mice. Mice fed an HFD had increased abundance of bacteria with capacity for lipid and fatty acid biosynthesis, that is, substrates which could become available as nutrients or bioactive compounds for the host and explain the observed increase in body weight and adiposity index in this group. The microbial composition of the HFD + Cal group was associated with increased activities of the phosphotransferase system, glycolysis, and gluconeogenesis compared to the HFD mice. These 2 functions enable the bacteria to take up and metabolize carbohydrates, producing metabolites which could potentially be used by the host. To determine if this is related to the reduced adiposity index in the mice fed oil from *C finmarchicus*, a further in-depth study is needed. Functional analysis of the microbiota from HFD + Ex mice showed upregulation of signal transduction pathways, but the implication of this observation also needs further studies.

This is the first study examining the effect of calanus oil, a wax ester–containing oil derived from *C finmarchicus*, on the gut microbiota composition. Obesity in female mice was associated with an enrichment of the proinflammatory *Lactococcus* and a depletion of the anti-inflammatory and health-promoting *Allobaculum* and *Oscillospira*, changing the microbiota composition in an unhealthy direction. Although dietary oil from *C finmarchicus* was not able to restore the microbiota composition to that of the lean control group, it resulted in a relatively high abundance of *Lactobacillus*, a health-promoting genus, which is often related to weight loss. It also reduced the abundance of the pathogenic *Bilophila* genus. Treatment with exenatide partly restored the bacterial profile found in the lean control group and seemed to reduce the abundance of *Streptococcus* and *Lactococcus*, which are both considered pathogenic. These data confirm our hypothesis, namely, that dietary supplementation with the antiobesogenic calanus oil is able to antagonize the unfavorable changes in the gut microbiota induced by high-fat feeding.

Differences in sample preservation and DNA extraction, library preparation, as well as use of different reference databases or software programs make it difficult to compare results from one laboratory to another [54-56]. In this study, the number of animals in each group was relatively small (n = 5), and hence, the power of the statistical analysis was relatively low. In addition, determination of the microbiota composition was based only on feces samples from the colon.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: TSL has a small position as scientific advisor in Calanus AS. AMP is employed as product manager in Calanus AS. PCS, KMJ, RLO, and JM declare no conflict of interest.

Supplemental materials

Supplemental materials were provided that include diet information; Table S1, Overview of all identified functional categories; Figure S1, Gene expression of markers related to inflammation, intestine permeability, and fat metabolism in the colon of female mice; and Figure S2, Gene expression of markers related to inflammation and fat metabolism in omental WAT of female mice. Supplementary data to this article can be found online at https://doi.org/10.1016/j.nutres.2020.09.002.

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Paper III

A simple method to isolate fatty acids and fatty alcohols from wax esters in a wax-ester rich marine oil

PLOS ONE

RESEARCH ARTICLE

A simple method to isolate fatty acids and fatty alcohols from wax esters in a wax-ester rich marine oil

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Abstract

Calanus finmarchicus is one of the most important zooplankton species in the North Atlantic. The zooplankton is currently being harvested and industrially processed to a marine oil product for human consumption as a marine nutraceutical containing long-chain omega-3 polyunsaturated fatty acids. This oil is very rich in wax esters, a lipid class where fatty acids are esterified to long chain fatty alcohols. In this paper we describe a simple method to 1) isolate the wax esters from the other lipid classes present in the oil, 2) hydrolyze the wax esters, and 3) separate the fatty acids from the fatty alcohol, all by means of solid phase extraction. Starting with an average of 322 mg Calanus oil, we obtained 75 mg fatty alcohols and 63 mg fatty acids. Contrary to previously described techniques, our method neither oxidize the fatty alcohols to fatty acids, nor are the fatty acids methylated, allowing the native, unesterified fatty acids and fatty alcohols to be used for further studies, such as in cell culture experiments to study the metabolic effects of these specific lipid fractions rather than the intact oil or wax esters.

Introduction

The limited amount of fish oil, containing health promoting long-chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA), available for the use in fish feed and human consumption, has led to a massive amount of research and development to find alternative sources for these fatty acids [1, 2]. Suggested possibilities have been to utilize lower trophic level alternatives, such as zooplankton, like krill and calanoid species, or the industrial cultivation of marine microalgae [3]. The marine copepod *Calanus finmarchicus* is one of the most important zooplankton species, with respect to biomass, in the North Atlantic and plays a key role in the pelagic food web between primary producers and economically important fish species [4–6]. Due to *C. finmarchicus*' importance in the marine ecosystem and the possibility to harvest it in a sustainable manner [7], the species has received substantial scientific attention. The zooplankton is currently being harvested and industrially processed to an oil (Calanus oil). [8, 9]. This oil, as most other marine oils, is a source of the health promoting LC n-3 PUFA [8, 9].

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However, unlike other marine oils, most of the fatty acids in Calanus oil (as much as 85%) are esterified to a fatty alcohol, forming a lipid class known as wax esters [8, 9].

Several analytical methods have been developed to identify and quantify the composition of the fatty acids and fatty alcohols in calanoid wax esters. These methods often contain transesterification, derivatization, separation, and purification steps of the wax esters and fatty acids and alcohols and lead to the production of fatty acid methyl esters (FAMEs) [10–13].

Previous studies have shown that the oil from *C. finmarchicus*, and especially the wax esters, have potent effects on gut microbiota and cardiac function and against the development of obesity, insulin resistance and inflammation in mice [14–18] and body composition, functional strength, cardiorespiratory function, metabolic markers and omega-3 index in humans [19–22], making this oil a possible new marine drug against metabolic syndrome and other pathological conditions.

After consumption, wax esters are to a large extend hydrolyzed to free fatty acids and free fatty alcohols in the lower end of the gastrointestinal tract [23]. The possible health effects of the wax ester derived fatty acids and fatty alcohols have been reviewed previouslyand it is known that both fatty acids and fatty alcohols can have positive health effects but potentially through different mechanisms [24]. To study the health effects of the individual lipid classes in the wax ester, in vitro, it is necessary to separate the fatty acids and fatty alcohols in hydrolyzed wax esters. To our knowledge there is no method previously described to separate the fatty acids and fatty alcohols in wax esters without the formation of FAMEs. Although the production of FAMEs is excellent for lipid composition analyses, it is less suitable for the extraction of specific lipid classes. FAMEs are esters of their own and would not permit us to obtain the free fatty acids without another saponification step. Similarly, the fatty alcohols would be oxidized to their corresponding fatty acids before being transformed to FAMEs. Thus, the production of FAMEs would not allow us to obtain the fatty alcohols and fatty acids in their native form without additional steps. Therefor the aim of this work was to develop a simple semi-preparative method using solid phase extraction (SPE), to isolate the wax esters from the other lipid classes present in the oil, and subsequently to separate the native non-esterified fatty acids and fatty alcohols in the wax esters. The method does not require sophisticated instrumentation. These isolated compounds may then be used to study potential metabolic effects in, for example, cell culture experiments.

Materials and methods

Oil and chemicals

The oil produced from *C. finmarchicus* (Calanus oil) was provided by the company Zooca® formerly named Calanus® AS (Tromsø, Norway). Heptane (99,8%), isopropanol (100%), diethyl ether (≥99,8%) and ethanol (96%) were purchased from VWR, Darmstadt, Germany. Acetic acid (99,8%) and hydrochloric acid (37%) were purchased from Honeywell, Seelze, Germany. Chloroform (99,0–99,4%), phosphoric acid 99,99%, potassium bicarbonate ≥99,95%, sodium hydroxide, copper (II) sulphate pentahydrate ≥98,0%, sulphuric acid and toluene were obtained from Sigma-Aldrich, Darmstadt, Germany. N-Hexane was obtained from Merck KgaA, Darmstadt, Germany.

Isolation of wax esters from Calanus oil

The wax esters were isolated from Calanus oil through solid phase extraction (SPE) as described by Vang *et al.* [9] with some modifications. On average 322 mg of Calanus oil were dissolved in 6 mL chloroform (Fig 1). A Mega Bond Elute (5g) aminopropyl SPE disposable column (Agilent Technologies, Oslo, Norway),used in all separation steps described in Fig 1,

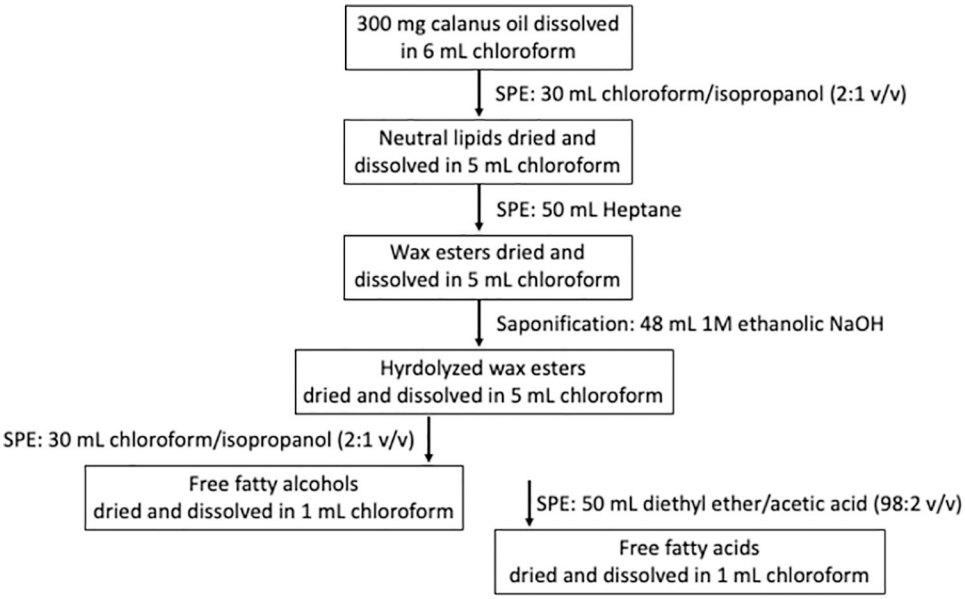

Fig 1. Isolation of fatty acids and fatty alcohols in wax esters present in Calanus oil by use of solid phase extraction. See text for details.

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was mounted on a SPE Visiprep vacuum manifold (Supelco, Bellafonte, PA, USA). The column was conditioned with 20 mL heptane at a flow rate of approximately 1 mL/min, a flow rate used in all separation steps described in Fig 1. After conditioning, the Calanus oil in chloroform was added to the column. The neutral lipids (NL) present in the oil were eluted with 30 mL chloroform/isopropanol (2:1 v/v) and dried under nitrogen gas. The dried NL were subsequently dissolved in 5 mL chloroform and then applied to a new SPE column pre-conditioned with 20 mL heptane. The wax esters were eluted with 50 mL heptane and again evaporated to dryness under nitrogen gas and dissolved in 5 mL chloroform. The yields of the eluted lipid classes were determined gravimetrically after drying beforethey were redissolved in chloroform to provide a sample for the HPTLC analysis. The isolation of wax esters and the subsequent saponification and isolation of the native fatty acids and fatty alcohols were carried out 3 times (see S1 Table).

Saponification of wax esters and isolation of the fatty acids and fatty alcohols

The isolated wax esters were saponified following Christie and Han [25] with slight modifications. The wax esters, on average 251 mg dissolved in 5 mL chloroform, were evaporated to dryness and dissolved in 50 mL of 1 M ethanolic NaOH to a concentration of 5 mg/mL. The dissolved wax esters were transferred to 5 GL 18 Duran Schott test tubes (DWK life sciences, Oslo Norway) each containing 10 mL dissolved wax esters in 1 M ethanolic NaOH, capped, and placed on a heating block (ThermoFisher Scientific, Oslo, Norway) for 90 min at 90˚C. No antioxidants were added during the wax ester hydrolysis because Calanus oil contains naturally the antioxidant astaxanthin. The hydrolyzed wax esters were cooled on ice and the Duran Schott test tubes were pooled before addition of 50 mL MilliQ water and 25 mL 6 M HCl. The acidified hydrolyzed wax esters were then mixed with 25 mL heptane to form two phases. The upper phase, containing the lipids, was collected and again evaporated under nitrogen gas and dissolved in 5 mL chloroform.

After the saponification, the free fatty acids and fatty alcohols were separated from each other by use of SPE (Fig 1). An SPE column was conditioned with 20 mL heptane after which the fatty acids and fatty alcohols in chloroform were added to the column. The fatty alcohols were eluted with 30 mL chloroform/isopropanol (2:1 v/v). The same column was then again conditioned with 20 mL heptane and the free fatty acids were eluted with 50 mL diethyl ether/acetic acid (98:2 v/v). The solvents with the separated fatty alcohols and fatty acids were evaporated under nitrogen gas and each dissolved in 1 mL chloroform. The use of SPE to separate the fatty alcohols from the fatty acids was chosen over the separation of the fractions via soap formation by NaOH, since the latter method did not lead to a clear separation in our set-up.

Analysis by HPTLC

From the first isolation, aliquots of 50 μL were removed from the initial Calanus oil in chloroform and the fractions eluted from the SPE columns (Fig 1); the neutral lipids, the wax esters, the hydrolyzed wax esters and the isolated fatty alcohols and fatty acids, all in chloroform. The aliquots were stored at -20˚C before analysis by high performance thin layer chromatography (HPTLC). One microliter of the samples was applied to the HPTLC plate (Silica gel 10 cm × 10 cm, Merck, Darmstadt, Germany) together with reference standard 18–5 A (Nu-Chek-Prep, Elysian, MN, USA). The plate was placed in a glass chamber saturated with heptane/diethyl ether/acetic acid (80:20:2 v/v/v) as described by Henderson and Tocher [26]. The mobile phase was allowed to migrate about 9 cm before the plate was removed and the solvent was allowed to evaporate. After air drying, the plate was sprayed with 10% copper sulphate in 8% phosphoric acid prior to development in an incubator at 180˚C for 3 min. The developed HPTLC plate was scanned on a Xerox WorkCenter 7855i (Fig 2).

Composition of the fatty acids and fatty alcohols

To analyze the composition of the wax ester, free fatty acids and fatty alcohols, aliquots of these lipid fractions from the first extraction round were dissolved in 1 mL toluene and 2 mL 1% sulfuric acid in methanol was added. The tubes were then flushed with nitrogen gas, capped, and incubated at 50˚C for 16 h. After cooling, 2 mL 2% $KHCO_3$ was added, followed by 10 mL freshly made n-hexane/diethyl ether (1:1 v/v). After centrifuging at 1500 rpm for 2 min, the organic layer was transferred to a clean tube. The aqueous layer was mixed for a second time with 10 mL n-hexane/diethyl ether (1:1 v/v) and centrifuged before the organic layer was transferred to the organic phase from the first extraction. The combined organic phases were then dried by evaporation under nitrogen gas. The final extract was dissolved in n-hexane, to a final concentration of 1 mg lipid per ml. The fatty alcohols and the methylated fatty acids were determined by gas chromatography analysis on GC-FID (Agilent Technologies GC 7890B, Santa Clara, CA, USA) and identified by use of the FAME standards GLC 68D and GLC 96, and the fatty alcohol standard GLC 621 (Nu-Chek-Prep, Elysian, MN, USA). GLC621 is a combination of C14:0 myristyl alcohol (15% wt/wt), C16:0 palmityl alcohol (15% wt/wt), C16:1 palmitoleyl alcohol (15% wt/wt), C18:1 oleyl alcohol (15% wt/wt), C20:1 11-eicosenol alcohol (10% wt/wt), C21:0 methyl heneicosanoate (20% wt/wt), and C22:1 erucyl alcohol (10% wt/wt). This analysis was performed by the company Akvaplan-niva, daughter company of the Norwegian Institute for Water Research (NIVA), Tromsø, Norway (https://www.akvaplan.niva.no/en), following an inhouse protocol based on methods described by Folch et al., [27] and Christie [28].

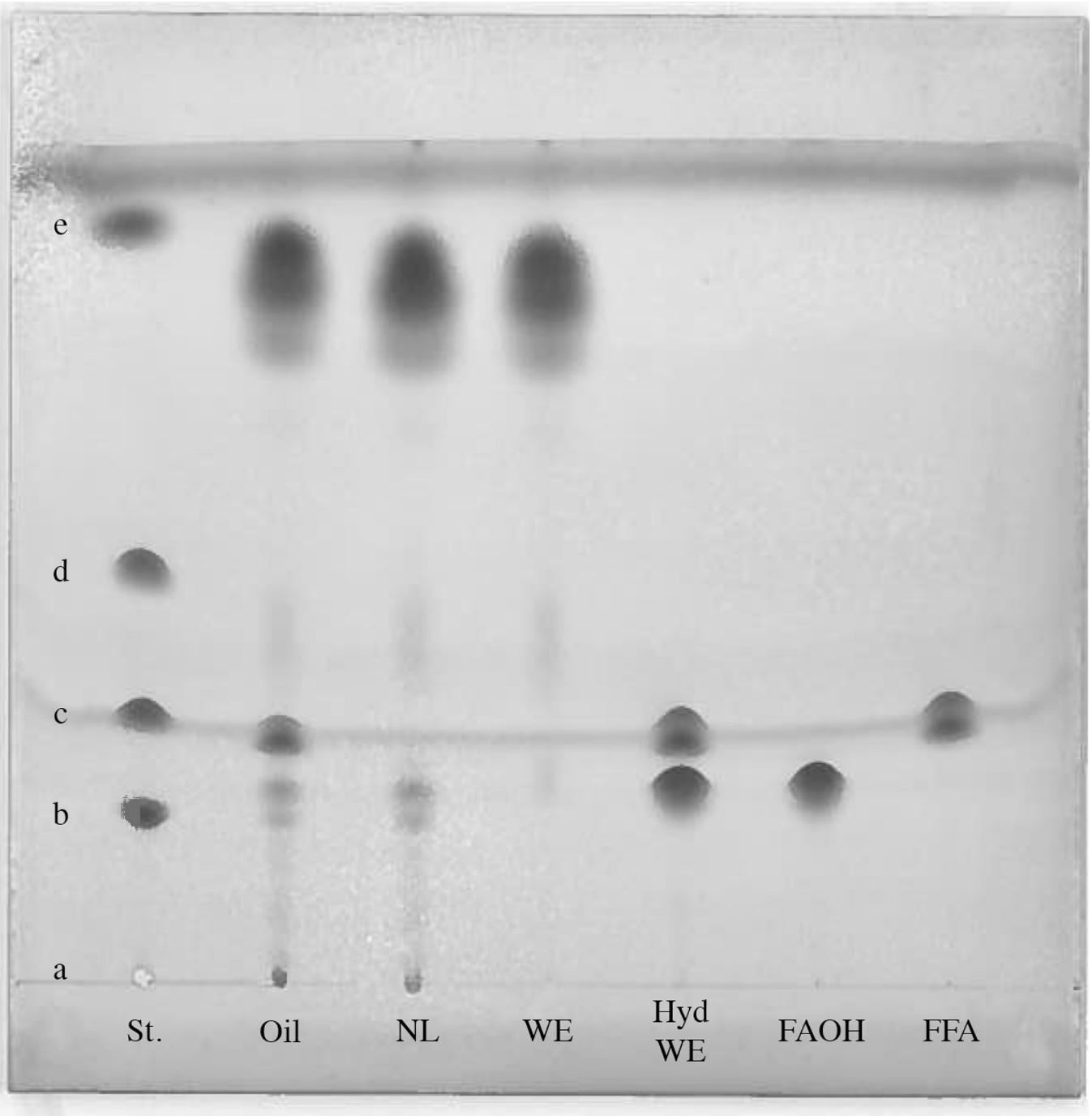

Fig 2. High performance thin layer chromatography of the lipid classes isolated from Calanus oil by SPE. St.: Lipid class standard 18–5 A containing lecithin (a); cholesterol (b); oleic acid (c); TAG (d); and cholesteryl oleate (e). Oil: Oil from *C. finmarchicus* (Calanus oil), NL: Neutral lipids, WE: Wax esters, Hyd. WE: Hydrolyzed wax esters, FAOH and FFA: free fatty alcohols and free fatty acids, respectively, from the hydrolyzed wax esters.

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Results and discussion

Wax ester isolation

A flow diagram of the different steps to isolate the fatty acids and fatty alcohols of the wax esters in the Calanus oil by means of solid phase extraction (SPE) is shown in Fig 1. The total weight of wax esters (WE) isolated from on average 322 mg Calanus oil was 251 mg (Table 1 and S1 Table), i.e. constituting almost 80% of the total lipids in the oil which is in the same range as found by others [6, 29]. According to the high performance thin layer chromatography (HPTLC) analysis (Fig 2), a relatively high amount of free fatty acids are present in the unfractionated oil and this has been reported previously [9]. It can also be seen that both the neutral lipids (NL) and WE fraction were devoid of free fatty acids. The 49 mg difference in weight between the oil (322 mg) and isolated neutral lipids (273 mg) is therefore most likely due to free fatty acid fraction and a small amount of phospholipids in the oil. The 22 mg difference between the neutral lipids (273 mg) and isolated wax esters (251 mg) is most likely due to the absence of cholesterol, mono- and diacylglycerol in the isolated wax ester fraction.

The migration of the WE fraction on the HPTLC plate is as described by Henderson and Tocher [26] using this solvent system. In initial SPE experiments, we found that most of the NL were eluted with only 10 mL chloroform/isopropanol (2:1 v/v) and only a small fraction was obtained with an additional 10–20 mL (see Fig 1 in S1 File). However, to maximize the yield we eluted the NL with 30 mL. Similarly, we eluted the WE with 50 mL of heptane although the majority were eluted with the first 10 mL (see Fig 2 in S1 File).

Saponification of wax esters and isolation of the fatty acids and fatty alcohols

The wax esters were hydrolyzed following Christie and Han [25] but using NaOH instead of KOH. Hydrolyses with KOH formed a soap like layer between the water and heptane layer that made it more difficult to recover the upper heptane layer containing the free fatty acids and free fatty alcohols. The method we used here showed to be effective in hydrolyzing the wax esters. In Fig 2, there are two clear separate bands visible, one corresponding to the free fatty acids and one band just below representing the free fatty alcohols. The free fatty acids band appears to consist of two bands with slightly different migration and this is probably due to different chain length and/or degrees of unsaturation of the different fatty acids present in the oil. No band is visible on the level of the wax esters, indicating that all wax esters were hydrolyzed.

After saponification, the free fatty acids and fatty alcohols present were separated from each other using SPE as shown in Fig 1 and described in the Materials and Methods section. A large fraction of the free fatty alcohols was eluted with the first 10 mL chloroform/isopropanol (2:1

Table 1. Average yield of different lipid classes extracted 3 times from Calanus oil by means of solid phase extraction. Values are given in mg and percentage weight of the oil with standard deviation.

Lipid class	Average (mg)	STDEV	Average %	STDEV
Calanus oil	322	19,1	100	
Neutral lipids	273	6,6	85	3,2
Wax esters	251	10,2	78	1,5
Hydrolyzed wax esters	153	10,1	48	3,6
Free fatty alcohols	75	7,4	24	3,5
Free fatty acids	63	6,5	20	3,2

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v/v). To extract all fatty alcohols, however 30 mL was needed (see Fig 3 in S1 File). From on average 322 mg Calanus oil a yield of 75 mg free fatty alcohols was obtained (Table 1).

After conditioning the column with 20 mL heptane, the free fatty acids were eluted with 50 mL diethyl ether/acetic acid (98:2 v/v). Most fatty acids are eluted with 40 ml heptane in which non are present in the first 20 mL (see Fig 4 in S1 File). From on average 322 mg Calanus oil, 63 mg free fatty acids could be recovered (Table 1). The HPTLC analysis clearly shows that the FAOH and FFA present in the hydrolyzed wax esters have been separated (Fig 2).

Composition of the fatty acids and fatty alcohols

Table 2 gives the fatty acid and fatty alcohol composition of the isolated wax esters and the isolated fatty acids and fatty alcohols after saponification. As expected, the results showed that almost

Table 2. Relative composition of the fatty acids and fatty alcohols (%) in the isolated wax esters from Calanus oil and the isolated fatty acids and fatty alcohols fractions after saponification of the wax esters from the first isolation.

Fatty Acids	Wax ester	Isolated fatty acid	Isolated fatty alcohol
14:0	9,8	19,7	n.d.
16:0	4,4	10,6	n.d.
18:0	n.d.	1,9	n.d.
16:1 n-7	2,6	5,2	n.d.
18:1 n-9	2,1	4,3	n.d.
20:1 n-7	0,9	n.d.	1,8
20:1 n-9	1,9	4,3	n.d.
20:1 n-11	0,4	0,8	n.d.
22:1 n-11	3,7	7,9	n.d.
18:2 n-6	0,6	1,2	n.d.
18:3 n-3	1,2	2,4	3,5
18:4 n-3	8,3	14,1	n.d.
20:3 n-6	1,5	0,8	2,6
20:4 n-3	0,8	1,3	n.d.
20:5 n-3	6,9	10,6	n.d.
22:5 n-3	n.d.	0,6	n.d.
22:6 n-3	3,5	4,5	n.d.
Σ SFA	14,2	32,2	n.d.
Σ MUFA	11,6	22,5	1,8
Σ PUFA	22,8	35,5	6,1
Σ Fatty acids	48,6	90,2	7,9
Fatty alcohols	**Wax ester**	**Isolated fatty acid**	**Isolated fatty alcohol**
14:0	0,6	n.d.	1,2
16:0	4,9	n.d.	9,1
16:1 n-7	1,2	n.d.	2,2
18:1 n-9	1,8	n.d.	1,6
20:1 n-9	12,6	n.d.	24,9
22:1 n-9	1,8	n.d.	2,4
22:1 n-11	22,5	0,8	46,0
Σ Fatty alcohols	45,4	0,8	87,4

Detection threshold is above 0,5%. n.d., not detected; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, poly unsaturated fatty acids.

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equal proportions fatty acids (48,6%) and fatty alcohols (45,4%) were found in the wax esters. Almost 80% of the identified fatty alcohols were monounsaturated, with eicosenol (20:1n-9) and docosenol (22:1n-11) being the dominating species. Of the polyunsaturated fatty acids, stearidonic acids (18:4 n-3), eicosapentaenoic acid (20:5 n-3) and docosahexaenoic acid (22:6 n-3) constituted about 30% of the fatty acids in the wax esters (Table 2). These results are similar to previous reported values [8, 9]. However, the composition of lipid classes and fatty acids in *C. finmarchicus* depends on the season, life cycle stage and geographic location [6, 30, 31]. It is therefore no surprise that the composition of the fatty acids and fatty alcohols in the wax esters found in this study is not exactly similar to the composition of the oil produced by the industry [8, 9]. The results from the fatty acid composition in the intact wax esters and the free fatty acid isolated from the hydrolyzed wax esters indicate that some oxidation may have occurred in the PUFAs, since the relative amount is lower in the isolated fatty acids than in the wax esters. Consequently, the relative amount of SFA has increased. Some oxidation may also have occurred in the MUFAs, but the relative amount is similar both in the intact wax esters and isolated fatty acids.

The established methods to analyze the oil in *C. finmarchicus* [11–13], focus on the determination of the composition of the lipids, rather than on extracting and separating the fatty alcohols and fatty acids for further use. Several methods have been described to extract policosanols from their natural sources, as reviewed by Shen *et al.* [32]. These methods include solvent extraction, transesterification and molecular distillation, supercritical carbon dioxide extraction, ultrasonic-assisted extraction, and saponification. As mentioned in the introduction, the previous described methods are excellent to study the fatty acid and fatty alcohol composition in calanoid species. But due to the production of FAMEs they are less suitable for the isolation of native, unesterified, fatty acid and fatty alcohol. It is possible to saponify the FAMEs to get free fatty acids. However, as can be seen in Table 1, the hydrolysis of the wax esters leads to a substantial weight loss from 251 mg to 153 mg. This leads us to suggest that an additional saponification step would further reduce the yield of the free fatty acids extracted from Calanus oil. To our knowledge, this is the first report of a simple, yet time consuming, method of semi-preparative isolation of the wax esters and the hydrolyzed components of wax esters, leaving the separated fatty acids and fatty alcohol unaltered. This allows them to be used for subsequent biological experiments.

Supporting information

S1 Table. Weight and percentage of Calanus oil weight of the extracted lipid classes from the 3 individual extraction rounds.
(PDF)

S1 File. Containing all supporting figures: Visualizations by means of TLC of the optimal elution volumes to extract the different lipid classes from Calanus oil.
(PDF)

S1 Raw images.
(PDF)

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Conceptualization: Pauke Carlijn Schots, Guro Kristine Edvinsen, Ragnar Ludvig Olsen.

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Project administration: Ragnar Ludvig Olsen.

Supervision: Guro Kristine Edvinsen, Ragnar Ludvig Olsen.

Writing – original draft: Pauke Carlijn Schots.

Writing – review & editing: Pauke Carlijn Schots, Ragnar Ludvig Olsen.

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Paper IV

Calanus oil wax ester-derived fatty acids protect H9c2 cardiomyoblasts from palmitate-induced lipotoxicity (Manuscript)