UiT
THE ARCTIC UNIVERSITY OF NORWAY

Faculty of Health Sciences, Institute of Medical Biology
Cardiovascular Research Group

Anti-Obesity and Anti-Hypertensive Action of Calanus Oil

Wahida Salma

A dissertation for the degree of Philosophiae Doctor – November 2014

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by

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Contents

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List of Papers

Paper I

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

Paper II

Höper AC, Salma W, Sollie SJ, Hafstad AD, Lund J, Khalid AM, Raa J,Aasum E, Larsen TS. Wax esters from the marine copepod *Calanus finmarchicus* reduce diet-induced obesity and obesity-related metabolic disorders in mice. J Nutr 2014,144 (2):164-9.

Paper III

Salma W, Franekova V, Lund T, Höper AC, Ludvigsen S, Lund J, Aasum E, Ytrehus K, Belke DD, Larsen TS. Dietary Calanus oil antagonizes angiotensin II-induced hypertension and tissue wasting in obese mice. Manuscript submitted in American journal of Physiology.

* Both authors contributed equally and shared first authorship.

Abbreviations

AA	arachidonic acid
ALA	α-linolenic acid
ApoB	apolipoprotein B
ATGL	adipose tissue triglyceride lipase
CCL2	chemokine C-C motif ligand -2
CLS	crown-like structures
DHA	docosahexaenoic acid
DIO	diet-induced obesity
ECM	extracellular matrix
ER	endoplasmatic reticulum
EMR1	EGF-like module-containing mucin-like hormone receptor-like 1
EPA	eicosapentaenoic acid
ET-1	Endothelin-1
eWAT	epididymal fat (white adipose tissue)
FA	fatty acid
FFA	free fatty acid
FAOH	fatty alcohol
GFAT	glutamine: fructose-6-phosphate aminotransferase
GLP-1	glucagon-like peptide-1
GLUT4	glucose transporter 4
GPR	G-protein coupled receptor
HBP	hexosamine biosynthesis pathway
HFD	high-fat diet
HIF1α	hypoxia-inducible factor 1-alpha
ICAM-1	inter-cellular adhesion molecule-1

IPGTT	intraperitoneal glucose tolerance test
IL	interleukin
IRS-1	insulin receptor substrate -1
JNK1	c-jun N-terminal protein kinse 1
LPS	lipopolysaccharide
MAP-kinase	mitogen-activated protein kinase
MCP-1	monocyte-chemoattractant protein-1
MIF-1	macrophage inflammation factor-1
MUFA	monounsaturated fatty acid
NEFA	non-esterified fatty acids
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NOS	nitric oxide synthase
OGA	O-GlcNAcase
OGT	O-GlcNAc transferase
OGTT	oral glucose tolerance test
PAI-1	plasminogen activator inhibitor-1;
PI 3-kinase	phosphatidylinositol 3-kinase
PDK-1	phosphoinositide dependent kinase-1
PKC	protein kinase c
PTM	post translational modification
PUFA	polyunsaturated fatty acid
pWAT	perirenal fat (white adipose tissue)
RAS	renin- angiotensin system
ROS	reactive oxygen species
SDA	stearidonic acid
SFA	saturated fatty acid

TAG	triacylglycerol
TNFα	tumor necrosis factor
Tx-A2	thromboxane A2
UPR	unfolded protein response
WAT	white adipose tissue
VCAM-1	vascular cell adhesion molecule-1
VPR	volume pressure recording

Preface

In this doctoral project we have examined the biological effects of Calanus oil in a mouse model of obesity. Calanus oil is extracted from the marine copepod *Calanus finmarchicus* and is one of the richest sources of poly-unsaturated fatty acids in nature.

C57BL/6J mice were given a high-fat diet (HFD) over a 27 wk period in order to induce obesity, which was reflected in deposition of considerable amounts of fat in the abdominal cavity and liver. In mice receiving HFD supplemented with a small amount of Calanus oil (1.5%) fat accumulation was significantly less, despite no difference in food intake between the groups. Obesity was also associated with increased expression of genes (mRNA level) coding for pro-inflammatory molecules, as well as macrophage infiltration in adipose tissue, indicative of a local (low-grade) inflammation. Moreover, insulin sensitivity was impaired as documented by reduction in glucose tolerance. These obesity-induced alterations were clearly antagonized by dietary Calanus oil, irrespective of whether it was given from the onset of the feeding period or after obesity was established. Another important finding was that wax ester from Calanus oil (i.e. the pure lipid component of the oil, devoid of antioxidants and other bioactive substances) was as effective as crude Calanus oil for obtaining these beneficial effects, and the efficacy of wax ester in producing the anti-obesity effect was even stronger than that of the clinically used ethyl esters of purified EPA and DHA. Finally, the increase in blood pressure which occurred when obese C57BL/6J mice were challenged by angiotensin II infusion was virtually abolished in mice that had been pre-treated with dietary Calanus oil. Dietary Calanus oil also antagonized the reduction in body and organ weights associated with angiotensin II infusion.

Collectively, these findings support the notion that low-grade inflammation in adipose tissue is the link between obesity and insulin resistance, and that reduction of visceral and ectopic fat mass by Calanus oil supplementation is an obvious strategy for targeting the inflammatory network. The capacity of dietary Calanus oil to antagonize angiotensin II-induced hypertension should also be ascribed to the anti-inflammatory action of the oil, both in the adipose tissue and vasculature.

Introduction

1. Obesity epidemic and obesity –related metabolic disorders

In the last 20 years the world has witnessed an alarming increase in obesity [1]. This global obesity pandemic is the leading cause for the soaring rates of metabolic diseases [2]. Today obesity (defined as a body mass index above 30) is prevalent in more than 34% of the adult population in the United States [3]. However, the condition is on an alarming rise also in the developing world, along with the adoption of a western life style [4]. According to the World Health Organization (WHO) 1.4 billion adults are overweight worldwide, and 500 million are obese. In near future these numbers are expected to rise unless effective actions are taken to prevent the development [5].

The current rise in human obesity is primarily linked to increased energy intake and decreased energy expenditure, resulting in excess fat deposition in adipose tissue [6]. There is considerable evidence indicating that obesity is a contributing factor for all major metabolic disorders, such as insulin resistance, diabetes and fatty liver disease, which in combination with cardiovascular disease and hypertension are collectively termed as metabolic syndrome [7], cardio metabolic risk [8] or multiple risk factor clustering syndrome [9]. Hence, there is a growing interest in the role of adipose tissue in the development of these pathologies [10]. Epidemiological studies show that visceral fat mass is more closely correlated with obesity-associated pathology than over all adiposity [11]. This includes the development of local and systemic chronic low-grade inflammation, characterized by increased infiltration of immune cells into adipose tissue and increased production and subsequent secretion of pro-inflammatory factors into the circulation [12].

1.1 Low grade inflammation in obese adipose tissue

Obesity, in particular abdominal obesity, is associated with a chronic local low-grade inflammation [6, 13, 14]. In this process the enlarged/expanded adipocytes start to secrete pro-inflammatory cytokines *(TNFα, IL-6, and IL-1β)* and chemokines, such as monocyte chemo-attractant protein-1*(MCP-1)* [15]. Macrophage infiltration occurs after initial rolling and

attachment of monocytes to activated endothelial cells. These monocytes then extravasate through the endothelial cell layer and differentiate into macrophages. Weisberg et al. [16] showed that chemokine C-C motif ligand -2 (CCL2) and its receptor, Chemokine receptor -2 (CCR2) play important roles in macrophage chemotaxis. At the onset of an inflammatory process, macrophages that are usually present in the adipose tissue switch from an anti-inflammatory (M2) state to a pro-inflammatory (M1) state [17]. Cross-talk between adipocytes, macrophages, and endothelial cells may aggravate the inflammatory state, resulting in increased secretion of pro-inflammatory cytokines (adipokines) and chemokines, as well as angiogenic factors. These factors could cause local and/or systemic insulin resistance in a paracrine and/or endocrine fashion, respectively, and might also induce local angiogenesis. More than 90% of M1-type macrophages are localized to dead adipocytes and form so-called "crown-like structures" (CLS), which is a characteristic immune-histological picture from adipose tissue both in obese mice and humans [18].

Numerous studies have shown that hypoxia and nutrient excess are the two main triggering factors for inflammation in adipose tissue [6, 19, 20]. In response to nutrient excess adipocytes expand and become hypertrophic. At the same time the distances between the blood bearing vessels increase and oxygen diffusion becomes insufficient [21], leading to local hypoxia. Thus, adipose tissue of obese individuals show decreased blood flow, increased vasoconstriction and reduced capillary density, compared to non-obese adipose tissue [20]. Hypoxia in the adipose tissue can also play a role in exacerbating pro-inflammatory cytokines and chemokines secretion by activating c-Jun N-terminal protein kinase 1 (JNK1) and IkappaB kinase/nuclear factor kappa B (IKK/NF- κB) pathways [13].

Philipp Scherer and co-workers [22] have documented increased interstitial fibrosis in white adipose tissue (WAT) during the development of obesity, which may reduce extracellular matrix (ECM) flexibility and decrease the tissue plasticity, ultimately leading to adipocyte dysfunction. Abnormal collagen deposition which is a hallmark of fibrosis development in adipose tissue, is closely associated with tissue inflammation and characterized by infiltration of macrophages and many other immune cells [23]. It has been reported that hypoxia inducible factor-1(HIF1α) is induced in response to fat pad expansion and induction of hypoxia. Under these conditions an entire set of “fibrotic response” genes are dramatically up-regulated, and classically activated pro-inflammatory M1 macrophages are attracted by dead adipocytes, which in turn lead to inflammation and metabolic dysfunction (Fig. 1) [22].

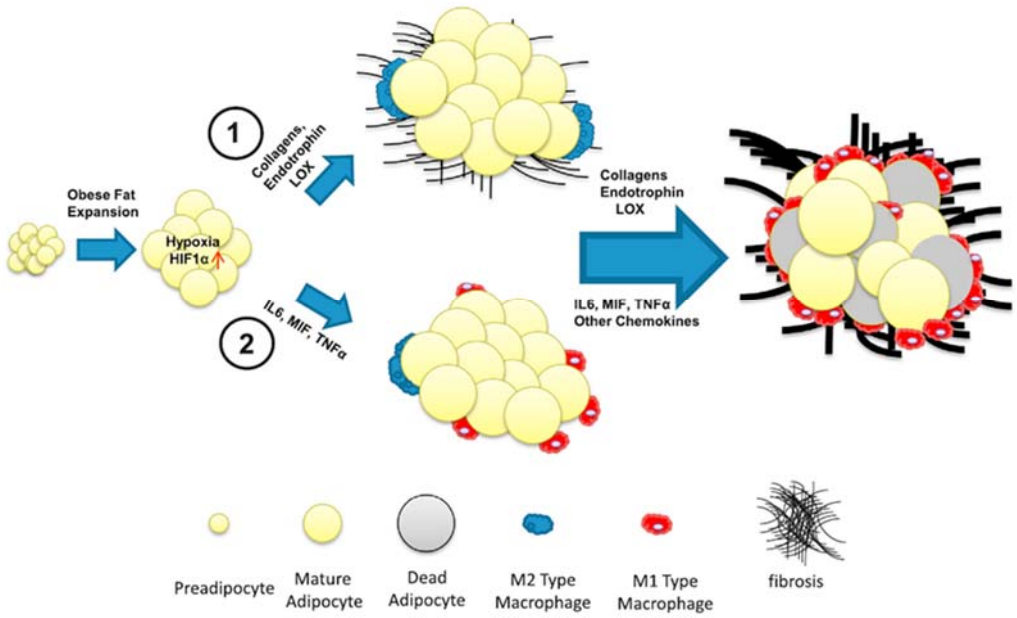

Fig. 1: Expansion of adipocytes in obesity leads to local hypoxia and activation of hypoxia-inducible factor 1-alpha (HIF1α), which in turn leads to upregulation of "fibrotic genes" and enzymes involved in collagen synthesis. This activation leads to local fibrosis and necrosis of adipocytes, causing M1 macrophage infiltration, inflammation and metabolic dysfunction. In addition, HIF1α may reinforce the inflammatory process by directly inducing pro-inflammatory factors, such as IL-6, TNFα and macrophage inflammation factor (MIF-1). From Sun K et al. [22] (with permission from Cell metabolism 2013, 18: 470-477)

1.2 Obesity and insulin resistance

Secretion of pro-inflammatory cytokines from obese adipose tissue leads to the development of a systemic inflammatory response which may impair peripheral organ function, including skeletal muscle, heart and vasculature [24, 25]. In the face of adipose tissue inflammation and fibrosis (as described above) the large ("fatter") adipocytes becomes dysfunctional with increased lipolytic activity. This response leads to increased release of free fatty acids (FFA) and ectopic fat deposition, which is believed to play a central role in the development of peripheral insulin resistance in both animals and humans [26, 27]. Thus, elevated FFA supply and ectopic lipid deposition can inhibit insulin-stimulated glucose transport through activation of various protein kinases (PKC, IKKβ and JNK) and attenuate expression of genes that are involved in mitochondrial oxidative phosphorylation , such as PPARγ co-activator‑1 (PGC‑

1). Inflammatory cytokines, such as TNFα, impairs insulin signaling, in part by inhibiting serine phosphorylation of insulin receptor substrate-1 (IRS-1) [28, 29], but also by inhibition of the insulin-regulated glucose transporter 4 (GLUT4) through activation of mitogen activated protein kinase kinase kinase kinase-4 (MAP4K4) and JNK kinases [27, 28].

Finally, it is believed that endoplasmic reticulum (ER) stress occurs during excess influx of nutrients, as well as during hypoxia, leading to activation of the unfolded protein response (UPR) [30]. Studies of insulin action on cultured rat liver cells show that increased activation of UPR leads to increased c-Jun N-terminal kinases (JNK) activity and Ser307 phosphorylation of IRS-1[31], linking ER stress and UPR up-regulation to insulin insensitivity and inflammation. It is also known that UPR increases IKKβ, which stimulates pro-inflammatory pathways [30, 32] , all leading to increased inflammation and insulin resistance.

Combination of obesity and insulin resistance often leads to the development of type 2 diabetes mellitus [33, 34], which is manifested by decreased insulin-stimulated glucose uptake and metabolism in skeletal muscle and adipose tissue, impaired suppression of hepatic glucose output [28, 35] and high levels of stored lipids in skeletal muscle.

1.3 Obesity and cardiovascular disease

The higher prevalence of cardiovascular disease in obese individuals associated with the increased frequency of various well known risk factors like hypertension, diabetes and dyslipidemia [36]. However, abdominal obesity with elevated production of pro-inflammatory adipocytokines and dysfunction of adipose tissue (described above) are key processes linking obesity to cardiovascular diseases, and are the fundament for the so-called "adipo-cardiovascular axis" [37]. Hence, abdominal obesity is regarded perhaps as the most serious new risk factor for metabolic and cardiovascular complications.

Many studies have demonstrated that isolated obesity in human subjects is associated with abnormal diastolic function [38], whereas impairment of systolic function is not consistently observed [39-41]. In humans, evidence suggests that obesity-related cardiomyopathy includes left heart remodeling (i.e., left atrial dilatation and left ventricular (LV) hypertrophy) as well as abnormalities in left ventricular contractile and relaxation functions [38]. Reduced LV systolic function has also been demonstrated in several animal models of obesity [42-45], except

for some studies in diet-induced obese rats, which showed an unchanged or mildly reduced or systolic function [46, 47]. Results from our own research group showed that high-fat diet enriched with sucrose resulted in reductions in both systolic and diastolic function in mice, as well as a marked concentric hypertrophy of the heart [48]. In addition, high-fat feeding results in a marked increase in myocardial oxygen consumption (due to increased oxygen cost for basal metabolism and excitation-contraction coupling) and a significant reduction in contractile efficiency [49].

1.4 Obesity and hypertension

An association between obesity and hypertension is well established in humans [50, 51]. A number of mechanisms have been suggested to be involved, including activation of the sympathetic nervous system, sodium retention, RAS activation, increased secretion of leptin and other neuropeptides, as well as insulin resistance and inflammation [52]. Damage and dysfunction of the vascular endothelium is an underlying factor in the pathogenesis of hypertension, and in the context of this thesis we will briefly mention how obesity-related insulin resistance and inflammation may impact on the endothelial function and produce hypertension.

Nitric oxide (NO), which is produced in the vascular endothelium from the amino acid L-arginine by the enzymatic action of the endothelial nitric oxide synthase (eNOS), plays a central role in vascular reactivity. Under normal conditions insulin phosphorylates and activates eNOS (via phosphorylation of IRS-1 and subsequent activation of PI 3-kinase, PDK-1 and Akt [53], resulting in increased NO production. In addition, the MAP kinase branch of insulin signaling causes secretion of vasoconstrictor endothelin-1 (Fig. 2).

The PI-3 kinase pathway is down-regulated, however, in insulin resistant states, leading to impairment of NO synthesis, whereas the MAP kinase pathway, on the other hand, remains unaltered. As a result, an imbalance between vasodilator and vasoconstrictor actions is established in favor of vasoconstriction [54]. In addition, elevated serum levels of glucose and FFAs impact negatively on endothelial NO production [55, 56].

Insulin-stimulated activation of NO production and inhibition of endothelin-1 production in vascular endothelium

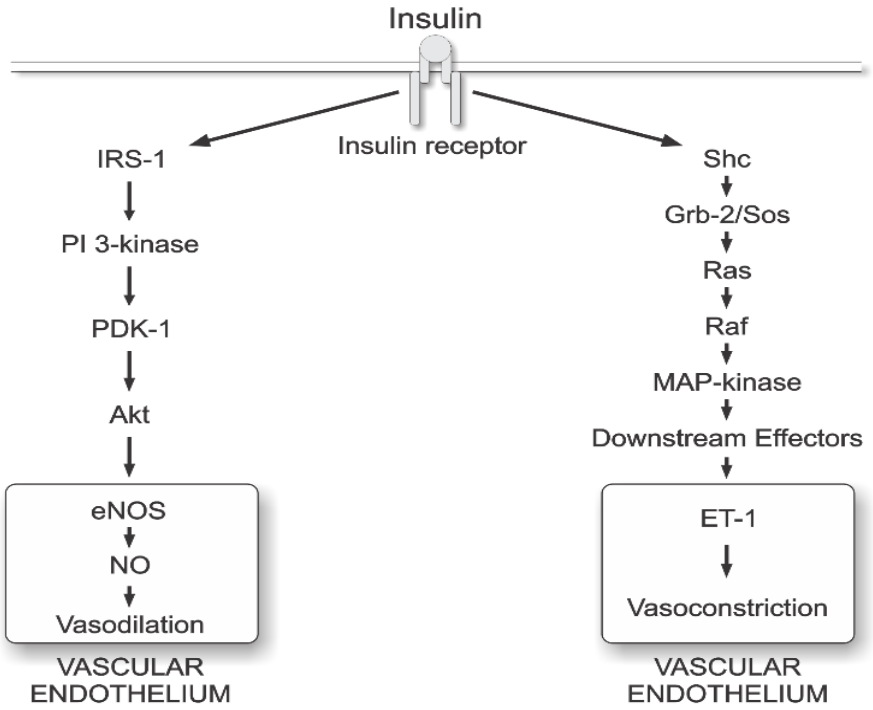

Fig. 2: Insulin receptor -1(IRS-1) binds and activates phosphatidylinositol 3-kinase(PI 3-kinase) which leads to phosphorylation and activation of phosphoinositide-dependent kinase-1 (PDK-1), which in turn phosphorylates and activates Akt. Akt phosphorylates and activates eNOS directly, resulting in increased NO production and vasodilation in vascular endothelium. On the other hand, insulin signaling via the mitogen-activated protein kinase (MAP kinase) regulates secretion of endothelin-1 (ET-1) and causes vasoconstriction in vascular endothelium. Adapted from Kim et al. [57].

A number of other biologically active molecules are also derived from obese adipose tissue (including perivascular adipose tissue) such as reactive oxygen species (ROS), vascular endothelial growth factor, plasminogen activator inhibitor-1, thromboxane A2 and acute phase reaction proteins (serum amyloid A proteins, C-reactive protein) [58, 59]. These compounds may impair NO production and lead hypertensions (Fig. 3).

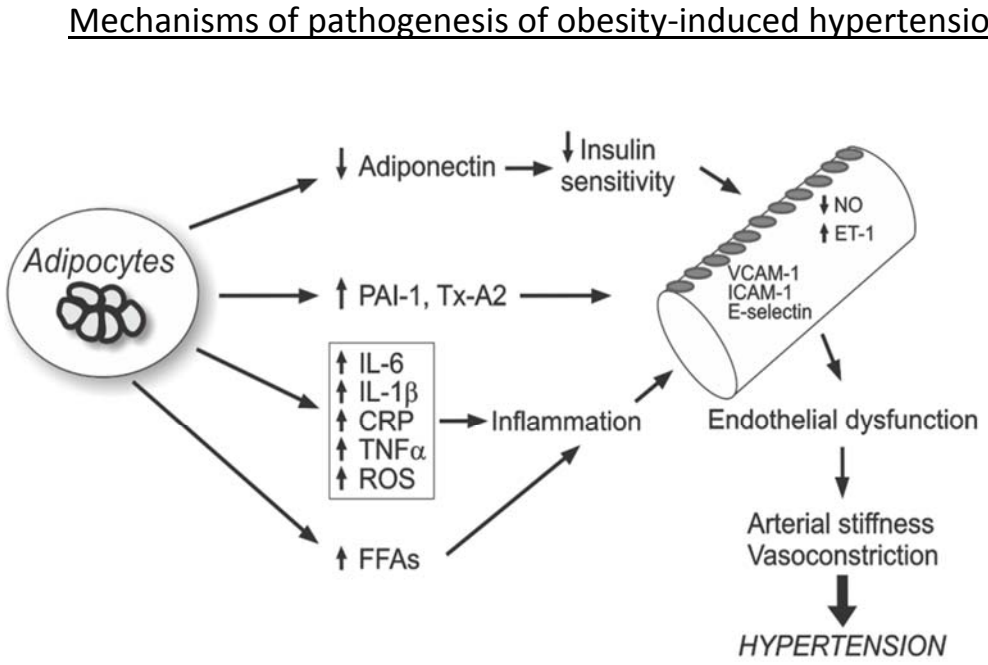

Fig. 3: Reduced adiponectin levels in response to obesity produce insulin resistance in vascular endothelial cells, which ultimately lowers nitric oxide (NO) production, while that of endothelin-1 is slightly increased. Enlarged adipocytes secrete pro-inflammatory cytokines, plasminogen activator inhibitor-1 (PAI-1) and thromboxane A2 (Tx-A2) and free fatty acids (FFA), which all contribute to endothelial dysfunction and hypertension. Adapted from Kotsis et al. [52].

1.5 Protein (O-GlcNAcylation) modification in obesity

Protein O-GlcNAcylation is a protein post translational modification (PTM) in which a single beta- acetyl-N-glucosamine moiety is attached to serine and threonine through formation of an O-linked ester, quite similar to protein phosphorylation. The level of protein O-GlcNAcylation can be regulated by glutamine: fructose-6-phosphate aminotransferase (GFAT), the rate-limiting enzyme in the hexosamine biosynthesis pathway or by the enzymes

catalyzing the addition or removal the O-GlcNAc moiety from proteins, i.e. O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) [60].

The nature of this PTM is controversial with respect to its impact on health, i.e. increased protein O-GlcNAcylation is linked to insulin resistance [61, 62], but it has also been shown to be cardioprotective when induced before a challenge such as ischemia-reperfusion, or oxidative stress whereas inhibition of O-GlcNAc formation decreases myocardial cell survival [63, 64]. It is generally agreed that that increased cellular O-GlcNAcylation lowers the phosphorylation of IRS1 Tyr608, decreases AKT activation and consequently decrease glucose uptake via GLUT4 [65]. By this mechanism, the nutritional status of the cell is intimately linked with the level of O-GlcNAcylation, nutrient processing, and insulin signaling [66].

An increase in protein O-GlcNAcylation has been shown to reduce inflammation and cytokine expression in relation to acute vascular injury [67] and has also been shown to preserve vascular reactivity in vessels exposed to elevated levels of TNFα [68]. The complete mechanism by which enhanced protein O-GlcNAcylation leads to a reduction in inflammation is unknown. Recent studies have suggested, however, that O-GlcNAc may act on transcription factors such as NFκB, to prevent their activation [69, 70]. In addition, it may reduce or mitigate the effects of ER stress, preventing further cell damage and apoptosis [71].

On the other hand O-GlcNAcylation has been shown to contribute to adverse effects of diabetes on the heart when glucose metabolism is increased via the hexosamine biosynthesis pathway (HBP) [72]. In addition, O-GlcNAcylation impaired cardiomyocyte hypertrophy and cell signaling pathways in diabetic models [72]. Lima and co-workers (2012) reported increased OGlcNAcylation in the vasculature in diabetes [73, 74], which could explain vascular dysfunction associated with arterial hypertension and diabetes [60].

2. Polyunsaturated fatty acids (PUFAs) and disease prevention

Polyunsaturated fatty acids (PUFAs) are fatty acids that contain more than one double bound in their backbone. The two main PUFA families, omega-3 and omega-6, have the final C-C double bond in the n-3 and n-6 position, respectively, and they are classified as essential fatty acids, since they cannot be synthesized in sufficient amounts and therefore need to be obtained via diet [75-77]. These fatty acids have important biological activities in cell function

and growth, reproduction [78] and regulation of gene expression [79]. Western diets typically contain high n−6/n−3 PUFAs ratio (15:1 to 16.7:1), which could promote inflammation and mediate of many chronic diseases, such as coronary heart disease, rheumatoid arthritis, obesity, diabetes, cancer, and mental illness [80, 81]. Moreover, there is evidence showing that a high content of n-6 PUFAs in the diet, comparative to n-3 PUFAs, is a predisposing factor for obesity [82]. Therefore, an optimal balance between n−6 PUFA/n−3 PUFA intake has been considered to be of importance when recommending PUFA supplementation for decreasing the risk of these chronic diseases [80, 83].Today the recommended intake of the essential n-3 and n-6 PUFAs ratio is 1:4-1:5 (75).

Linoleic acid (18:2, n−6) is a representative of the n−6 PUFA family. It is very abundant in the western diet, and is the precursor of arachidonic acid (20:4, n−6) (76). Alpha linolenic (18:3, n−3), which is found in vegetable oils, is the common precursor of eicosapentaenoic acid (EPA, 20:5, n−3) and docosahexaenoic acid (DHA,22:6, n−3) which are the typical marine n-3 PUFAs. Both n-3 and n-6 PUFAs can compete for the same enzyme for elongation and desaturation in the metabolic pathway (Fig. 4). So, if there is an excess of one fatty acid family it can interrupt the metabolism of the other [84].Therefore, an excessive intake of linoleic acid lowers the formation of EPA and DHA, the two main n-3 PUFAs [85].

Polyunsaturated fatty acid biosynthesis

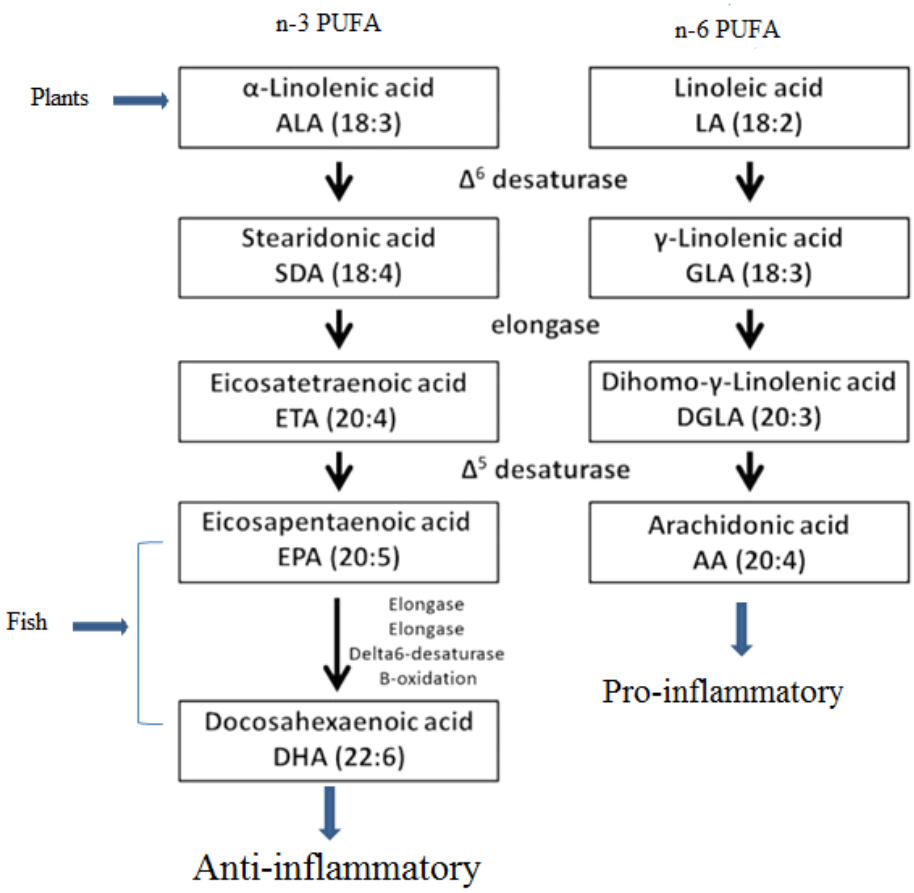

Fig. 4: The two essential fatty acids α-linolenic acid (ALA) and linoleic acid (LA) are abundant in seeds and vegetable oils. They are metabolized to produce PUFAs, where the initial step is the addition of a double bond to both ALA and LA to form the respective desaturated products. These desaturated metabolites are elongated and another desaturase can add a double bond to these elongated products to produce EPA and AA, respectively. EPA is converted into DHA through a series of enzymatic steps. Adapted from Tourdot BE et al. [86].

2.1 Anti-inflammatory action of n-3 PUFAs

Both animal and human studies have shown that supplementation of EPA and DHA may be protective against obesity, and may lessen weight gain in already obese animals and humans [87]. Thus, in rats fed with high lipid diets combined with n-3 PUFAs [88, 89] the amount of visceral (epidydimal and/or retroperitoneal) fat was reduced in a dose-dependent manner [90].

The reduction in visceral fat was seen in some studies without changes in energy intake [88, 91, 92] while other studies reported a significantly decreased food intake [93, 94].

One of the advantages with increasing n-3 PUFAs in the diet is related to its anti-inflammatory action [85]. There is a variety of molecular mechanisms underlying the anti-nflammatory action of n−3 PUFA, namely altered synthesis of eicosanoids (prostaglandins, leukotrienes), activation or inhibition of nuclear receptors (e.g. peroxisome proliferator-activated receptor γ [PPARγ], liver X receptors) and alterations of membrane lipid rafts [95, 96]. There is no evidence for a selectivity of PPARγ for n−3 PUFA [97] regardless of anti-inflammatory effects of PPARγ agonists in obese animals and humans [98, 99]. Other suggested mechanisms behind n-3 PUFA-mediated reduction in inflammatory activity include inhibition of the pro-inflammatory NFkB signalling pathway, increased production of pro-resolving mediators (such as resolvins, protectins or maresins) [100] and activation of the novel G-protein coupled receptor (GPR) 120 [101, 102].

2.2 n-3 PUFAs and cardiovascular diseases

For many years it has been clear that dietary inclusion of marine oils rich in PUFAs exerts anti-atherogenic actions in human coronary heart disease [103-105]. The beneficial effects of n-3 PUFA on the cardiovascular system might result from their effects on some modifiable risk factors such as plasma lipids [77, 106, 107] and blood pressure [108-110]. A meta-analysis by Morris et al. demonstrated a significant and dose-dependent hypotensive effect of fish oil in patients with hypertension [111]. The cardioprotective effects of n-3 PUFA are supported by a number of experimental studies in cell culture [112] and animal studies [108, 113], as well as human trials [85, 114]. Population studies have recommended that regular fish meals (especially fat fish) could protect against many cardiovascular events [106, 115]. This effect has been associated with the high content of n-3 PUFA. n-3 PUFA in the form of fish oil or its purified constituents, EPA and DHA has been shown to be inversely related to cardiovascular mortality [114, 116, 117]. Thus, numerous studies have reported that n-3 PUFA lowered risk factors for heart failure, such as obesity, type II diabetes, insulin resistance, hypertension and inflammation [118-122].

The mechanism by which n-3 PUFA reduces blood pressure is still to be determined. However, dietary supplements with PUFAs have been shown to prevent hypertension in

various animal models by increasing nitric oxide production , decrease vascular wall thickness in the coronary arteries as well as blunting renin-angiotensin-aldosterone system and modulation of calcium release in smooth muscle cells [108, 123, 124]. Alterations in vascular reactivity and reduction in Serum thromboxane A2 (TXA2) level upon fish oil supplementation has been suggested to possible mechanism for the hypotensive effect [113]. A few studies have suggested that n-3 PUFA influences blood pressure through an interaction with angiotensin II [125]. In addition, anti-inflammatory and blood pressure-lowering properties of these fatty acids might provide protection of the cardiovasculature [85]. Thus, administration of n-3 PUFA lowered blood pressure in AngII -induced hypertensive rats [126]. Eicosapentaenoic acid (EPA) has been shown to depress vascular responses induced by exogenous Ang II in rabit [127]. In humans moderate doses of fish oil could reduce vascular resistance in response to infusion of Ang II [128, 129].

3. Calanus oil-a novel marine oil

Calanus oil is extracted from the marine copepod *Calanus finmarchicus*, which is the most abundant crustacean and one of the dominating food sources for fish in the North Atlantic [130]. The copepod is small (3-4 mm long) and grows in large volumes (200-400 tons) in the sea masses, providing approximately 50% of annual biomass production on the North Atlantic [131]. The copepod nourishes itself on various forms of phytoplankton, and during the spring and summer months it stores large amounts of energy in the form of oil, which can account for as much as 50% of its dry weight. During the fall and winter the copepod sinks to depths from 500-2500 m where it "hibernates" until the nest spring when it returns to surface waters. *Calanus finmarchicus* does not accumulate environmental toxins, as it is situated at a very low trophic level of the marine ecosystem. Therefore, the oil can be used in its natural form, avoiding cleaning processes, which is normally required for marine oils from fish and sea mammals [132].

Like other marine oils, Calanus oil is very rich in the essential marine n -3 fatty acids EPA and DHA (Table 1). Calanus oil also contains the essential fatty acid stearidonic acid (SDA, 18:4, n-3) and other long chain mono-unsaturated fatty acids (MUFA), such as gondoic acid (20:1 n-9) and cetolieic acid (22:1 n-11) [133]. Table 1 shows the composition of Calanus oil (average of >3 batches of oil harvested in different years) [92].

Table 1. Concentrations of the main components of Calanus oil

	g/100g oil
Saturated fatty acid (SFA)	14.7
Mono unsaturated fatty acid (MUFA)	14.0
Poly unsaturated fatty acid (PUFA)	
n-3 PUFA	19.2
n-6 PUFA	1.2
Fatty alcohol (FAOH)	39
Sterols	0.5
Astaxanthin	0.1
Others	11.3

The fatty acids in Calanus oil is mostly bound as monoesters (also known as wax esters), where the fatty acids are linked to long-chain unsaturated alcohols. In krill the larger part of the fatty acids are bound in phospholipids, while in in fish and marine mammals they are bound as triglycerides.

Calanus oil also contains proteins, vitamins, minerals, phytosterols, as well as a high amount of the antioxidant astaxanthin. The astaxanthin is giving the oil its characteristic red color, and it is one of the strongest anti-oxidant found in nature [134]. In addition to protecting the stored lipid in calanus finmarchicus [135], astaxanthin is suggested to have potential health benefits in cancer, chronic inflammatory and neurodegenerative conditions, as well as in cardiovascular- and metabolic diseases [136].

Objectives

Lipids of marine origin have received considerable attention, because of their beneficial effects on cardiovascular health (anti-inflammatory, anti-thrombotic, anti-arrhythmic, hypo-lipidemic and vasodilatory action). Oil from the marine copepod *Calanus finmarchicus* has a unique composition, and in a recent study, Eilertsen et al.[137] showed that dietary supplementation with Calanus oil was able to decrease plaque formation in apoE-deficient mice.

This doctoral project was designed to further examine the biological effects of Calanus oil, using a mouse model of diet-induced obesity. The specific objectives were:

1. to investigate the effect of Calanus oil on diet-induced (abdominal) obesity and its metabolic disorders, using both a preventive and a therapeutic approach
2. to determine if purified wax ester from Calanus oil could provide the same effects as crude Calanus oil, i.e. whether potential beneficial effects of the lipids remain in the absence of other biologically active constituents of the oil
3. to determine the impact of Calanus oil on hypertension and cardiac remodeling in diet-induced obese mice challenged with two weeks of Ang II infusion to induce a cardiovascular stress.

Methodological considerations

1. Animals and dietary regimens

In this project we used a mouse model of diet-induced obesity. The animals were housed at Department of Comparative Medicine (Faculty of Health Sciences, UiT The Arctic University of Norway) and treated according to the guidelines on Accommodation and Care of Laboratory Animals Used for Scientific Purposes Formulated by the European Convention for the Protection of Vertebrate Animals. All procedures were approved by the local authority of the National Animal Research Authority in Norway.

Obesity was induced by feeding male C57BL/6J mice a lard-based high-fat diet (HFD) (Test diet 58V8, corresponding to the original D12451 from Research Diets) containing 18, 36 and 46% of energy from protein, carbohydrate and fat, respectively. Because of it relatively high content of carbohydrate this diet resembles a typical "Western" type diet. Lean control mice were given normal chow containing 18, 72 and 10% of energy from protein, carbohydrate and fat (CTR, no. 58Y2, Test Diet; IPS Limited). The specification sheets of the diets are shown in the Appendix.

It should be noted that addition of Calanus oil (1.5 g/100 g) to the HFD was compensated for by the removal of the same amount of lard, so that the total fat content was similar and the diets remained isoenergetic. It is also worth noticing that the amount of Calanus oil was only a fraction of that used in similar studies reported in the literature, and there was no indication that the animals did not like the food, since we the food intake was the same for the groups receiving HFD with and without Calanus oil.

C57BL/6J mice is a common inbred laboratory mouse strain, meaning that the genotype of the individuals within this strain is nearly identical, which will reduce individual variation and increase the likelihood for detecting significant differences between experimental groups. In addition, animal studies have the advantage that they can be performed under strictly controlled laboratory conditions (temperature, humidity etc.), which also will reduce individual variability. C57BL/6J mice are regarded obesity-prone, and during high-fat feeding they develop many of the same characteristics as found in human obesity, such as elevated plasma glucose, insulin resistance and ectopic fat deposition [138, 139]. This mouse strain, therefore, is regarded as a suitable model for studying pathophysiological consequences of

obesity. Results obtained in mice should, however, be extrapolated to humans with caution, because humans are quite heterogeneous genetically, and the effect of a certain treatment could vary considerably – also because of differences in e.g. digestive physiology and metabolic regulation.

2. Analytical methods

2.1 Glucose tolerance test

The global incidence of obesity and type 2 diabetes requires new therapies for treatment. The diet-induced obese mouse model has been metabolically well characterized, and several methods are used for assessment of glucometabolic control in this model. The oral glucose tolerance test (OGTT) is considered the most physiological test, since it mimics the normal route by which carbohydrates are ingested. Following absorption from the intestinal tract and uptake in the splanchnic and systemic circulation, blood glucose concentration increases. The elevation of blood glucose is in turn a major stimulus for insulin release from the pancreas. The passage of carbohydrates through the first part of the intestine stimulates the release of the gut hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which in turn augment the beta cell sensitivity to glucose, increasing the production of insulin [140, 141].

During an OGTT glucose is administered by means of a gavage tube. This procedure can imply stress to the animal and unreliable glucose values, and for this reason we used a simple intra-peritoneal glucose tolerance test (IPGTT). Glucose was injected intra-peritoneally, and the mice were placed in a restraining cage, allowing for repeated blood sampling from a small incision of the saphenous vein. The gut effect (i.e. glucose-stimulated GIP and GLP-1 release) is however lost by the use of this method.

The hyperinsulinemic –euglycemic glucose clamp technique provides an absolute index of insulin sensitivity. It is used mainly for humans and large animals, but in recent years it has also been adopted for mice [142, 143]. It is a relatively time-consuming and requires good technical skills and that the animals are anesthetized.

2.2 Fat depots in abdomen

Adipose tissue was for many years regarded merely as a passive energy store, but our present understanding is that adipose tissue has important endocrine functions, by secreting several immune-modulatory proteins (adipokines). Moreover, obesity is associated with a local low-grade inflammation in adipose tissue, as reflected by an increased expression of genes coding for pro-inflammatory adipokines and diminished expression of anti-inflammatory adipokines. The resulting adipokine imbalance is suggested to play a key role in the development of obesity-related metabolic dysfunction and cardiovascular disease [144].

Local hypoxia has been suggested as the triggering event for adipokine expression in obesity, due to the fact that diffusion of oxygen becomes limited as the adipocytes grow in size [145, 146]. We were therefore interested in finding out whether Calanus oil could prevent fat deposition (adipocyte expansion) during high-fat feeding. Visceral or intra-abdominal fat (including omental, mesenteric, perirenal and perigonadal fat) is considered a major source of pro-inflammatory adipokines, and abdominal obesity is more closely related to metabolic dysfunction and cardiovascular disease than general obesity [11, 147]. In addition, it has been reported that insulin sensitivity in rodents can be improved by surgical removal of epididymal (eWAT) and perirenal (pWAT) [148, 149]. In our hands, we found it relatively easy to identify and dissect out pWAT and eWAT, and that we could determine the mass of these with high accuracy. Perirenal fat mass was normally chosen as an indicator of abdominal obesity. In addition, this depot was used for immune-histological examinations.

2.3 Hepatic triacylglycerol content

In addition to adipose tissue, the liver is affected by obesity, and inflammatory gene expression increases in liver with increasing adiposity [150], creating an inflammatory response similar to the adipose tissue inflammation that follows adipocyte lipid accumulation. The pro-inflammatory cytokines activate a number of immune cells present in liver, in particular Kupffer cells, which are believed to participate in hepatic inflammation [150, 151]. It was therefore of interest to determine whether high-fat feeding resulted in increased triacylglycerol content in the liver and even more pressing, whether dietary Calanus oil could the attenuate any hepatic fat deposition.

Triacylglycerol content in liver tissue can be determined by biochemical or histological techniques. We used a biochemical method based on enzymatic degradation of the triacylglycerol molecules to yield free fatty acids and glycerol. Since the amount of fat may vary depending on the region where the biopsy is taken, we homogenized a relatively large piece of liver tissue. Lipids were extracted from aliquots of this homogenate, using the method of Folch [152]. The extracted lipids were dried and emulsified in a special buffer before the chemical analysis (measurement of glycerol).

2.4 Immunohistological investigations

In our studies we identified macrophages by staining with the antibody F4/80 which is an extracellular antigen (glycosylated proteoglycan) on murine macrophages [153] similar to human EMR1 (EGF-like module-containing mucin-like hormone receptor-like 1) which is encoded by the EMR1 gene. The F4/80 marker is used as a general macrophage marker, but CD11c (M1) or MGL-1 (M2) can differentiate between M1 and M2 sub populations [17, 154]. Preferably we would have used one or several of those more specific antibodies. However, using F4/80 antibody dead adipocytes surrounded by macrophages (so-called crown like structure (CLS)) appeared clearly in our immunohistochemistry sections, and therefore we considered the choice of the F4/80 antibody to be acceptable for our analysis.

2.5 Real time quantitative PCR (qPCR)

Real-time qPCR is an important tool in gene expression analysis and has gained acceptance because of its rapidity and sensitivity as compared to the traditional method for quantitative measurement of gene expression, such as Northern blotting. Both absolute and relative quantification of gene expression can be analyzed by Real-time qPCR. It is important to choose a suitable gene for use as a reference or housekeeping gene when performing relative quantification of the expression of a target gene. The expression of the housekeeping genes should not vary in response to the experimental intervention but, unfortunately, housekeeping genes can still vary despite being constant in a given cell-type or experimental condition [155].Therefore, in our study we used the geometric mean of the three best out of a selection of

5-6 housekeeping genes, instead of using one reference gene as recommended by Vandesompele et al.[155].

2.6 Blood pressure measurement

Blood pressure was measured in conscious animals using the tail-cuff method (Kent Scientific, CODA- Torrington, CT, USA). This Non-invasive tail-cuff blood pressure device utilizes Volume-Pressure Recording (VPR) and is regarded as a valuable tool for measuring systolic and diastolic blood pressure in high-throughput experimental designs. Feng et al. [156] validated the VPR tail-cuff method by comparison to the more accepted radio-telemetry method and concluded that it provides accurate blood pressure measurements over the physiological range in mice. Furthermore, this method offers the highest degree of correlation with telemetry and direct blood pressure measurements, and it is clearly the preferred tail-cuff sensor technology [157]. In addition, the methodology requires no surgery, and it is significantly less expensive than other blood pressure methods. It should be noted that obesity in rodents is not always accompanied by hypertension [158, 159] and in order to get a "window of treatment" we decided to raise blood pressure by Ang II infusion.

3. Study Design

Paper I

Diet-induced obese mice were obtained by feeding 5–6-week-old C57BL/6J male mice (Charles River) a lard based high-fat diet (HFD, no. 58V8, Test Diet; IPS Limited) containing 18, 36 and 46% of energy from protein, carbohydrate and fat, respectively .There were three groups of obese mice: the first receiving the high fat diet (HFD) throughout the whole 27-week feeding period ; the second receiving the HFD supplemented with 1.5% (w/w) Calanus oil from the start and throughout the entire 27-week feeding period (preventive treatment, CAP); the third receiving the HFD (without supplementation) for 7 weeks, followed by the HFD with 1.5% (w/w) Calanus oil supplementation for the remaining feeding period (therapeutic treatment, CAT) (Fig. 5). It is important to note that addition of Calanus oil was compensated for by the removal of 1.5 g lard/100 g diet, so that the total fat content was unchanged and the diets remained isoenergetic. Body weight was recorded weekly throughout the experimental period, while food (energy) intake and glucose tolerance were recorded towards the end of the period. Tissue samples for biometric, immunohistochemical, and gene expression analysis, as well as blood samples for biochemical analysis, were sampled at sacrifice.

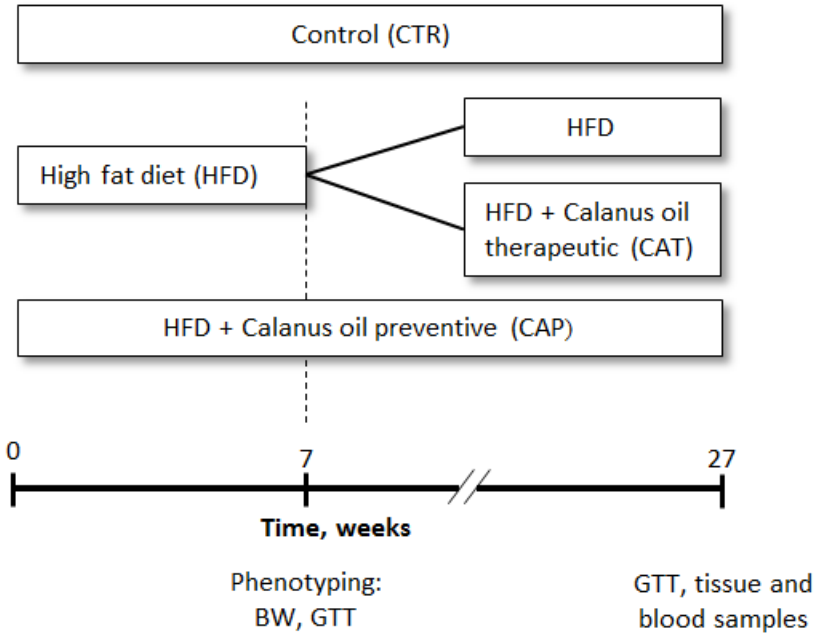

Fig. 5: Dietary regimen, time course and experimental interventions for paper I.

Paper II

The experimental design followed pattern as in paper I. Again we used 5-6 wk old C57BL/J6 male mice (Charles River) which were fed an HFD (catalog no. 58V8; TestDiet, IPS Ltd.). After 7 weeks, however, the mice were divided into 3 groups, receiving either HFD alone , HFD supplemented with 0.2% (wt:wt) purified EPA + DHA ethyl esters (OMACOR; Pronova BioPharma) or 1% (wt:wt) Calnus oil-derived wax ester (Fig. 6) . The amount of EPA and DHA added to the HFD was equivalent to the total content of n–3 PUFAs in the wax ester supplemented diet. This dietary regimen was continued for another 20 weeks.

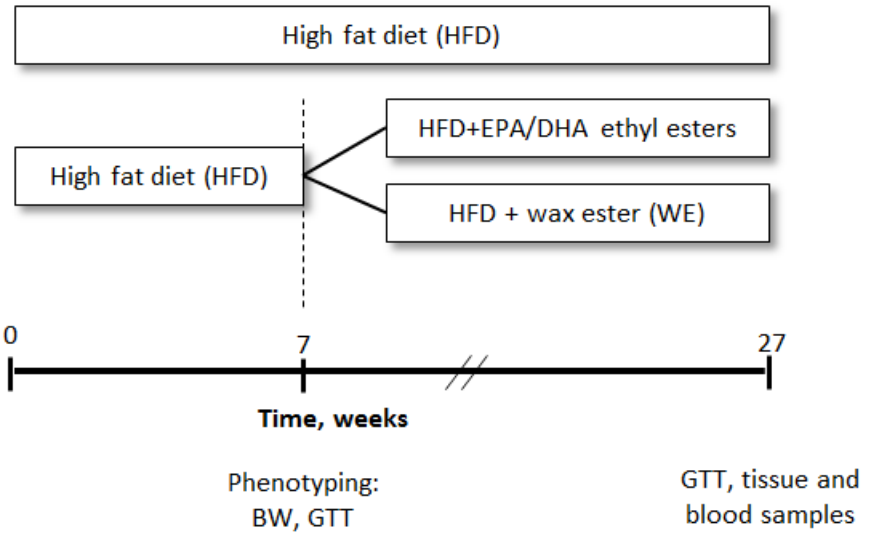

Fig. 6: Dietary regimen, time course and experimental interventions for paper II.

Paper III

C57BL/J6 male mice (5-6 week old at the start of the feeding period) were randomly divided in two groups, one receiving HFD supplemented with 2% (w/w) Calanus oil (HFD+CAL), while the other received no supplementation (HFD). After an initial 8 week feeding period, both groups were further sub-divided into two groups, receiving Ang II (Calbiochem, Dramstadt , Germany) (1µg/kg/min) or saline for another two weeks via mini osmotic pumps (Alzet mini osmotic pump) (Fig. 7), while on the same dietary regimen. Body weight and blood pressure were measured weekly during the initial 8 weeks and 3 days/week after Ang II

administration. Tissue samples for biometric, immunohistochemical, and gene expression analysis, as well as blood samples for biochemical analysis, were sampled at sacrifice.

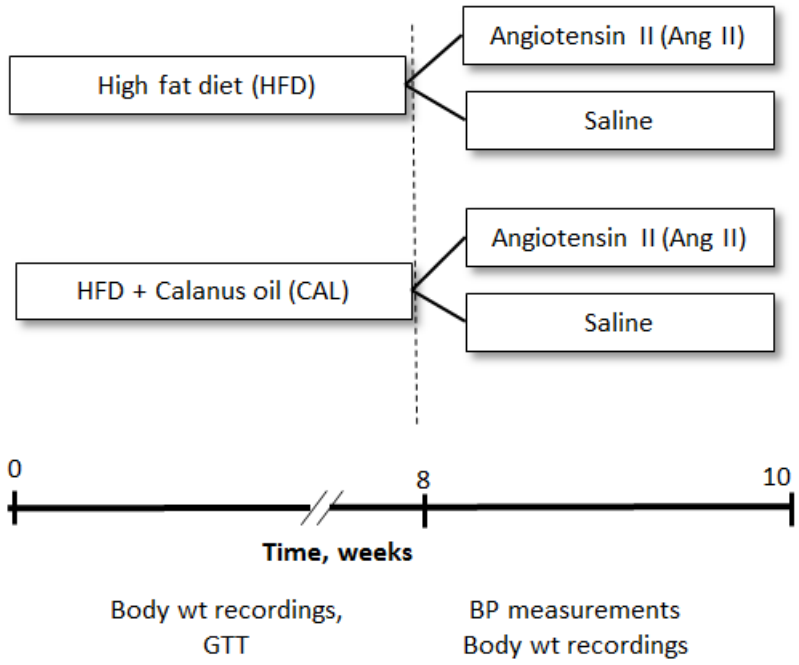

Fig.7: Dietary regimen, time course and experimental interventions paper III.

Summary and main results

Paper I

The main finding of this study was that dietary supplementation with Calanus oil significantly reduced body weight gain, abdominal fat deposition and hepatic steatosis in high-fat fed C57BL/6J mice. At the same time it improved insulin sensitivity, as determined by a glucose tolerance test. It should be noted that these effects were obtained by dietary concentrations of n-3 fatty acids which were considerably lower than those reported to attenuate obesity and obesity-related abnormalities in previous studies. Calanus oil supplementation also reduced adipocyte size, macrophage infiltration and mRNA expression of pro-inflammatory cytokines *(TNFα, IL-6 and MCP-1)* in abdominal fat depots, while mRNA expression of adiponectin was increased. Moreover, the effects of Calanus oil were not only preventive, but also therapeutic, as the oil proved to be beneficial, regardless of whether supplementation was started before or after the onset of obesity and glucose intolerance. Although this study did not focus on the mechanism(s) by which Calanus oil provides its beneficial effects, we anticipated that they could be ascribed to the n-3 fatty acids EPA and DHA and/or antioxidants in the oil.

Paper II

A major aim of the second study was to find out if dietary supplementation with Calanus oil-derived wax ester could mimic the biological effects of crude Calanus oil. In addition, we wanted to compare the effect of wax ester with ethyl esters of purified EPA and DHA. Thus, we used C57BL/6J mice which received a high-fat diet, starting supplementation with wax ester or EPA and DHA ethyl ester after obesity and glucose intolerance was established. The results obtained with wax ester supplementation was almost identical to those obtained with crude Calanus oil in study I, i.e. reduced body weight gain, reduced abdominal fat and hepatic steatosis, while glucose tolerance was improved. In adipose tissue, macrophage infiltration was significantly reduced, mRNA-expression of proinflammatory genes *(TNFα, IL-6 and MCP-1*) downregulated and adiponectin expression upregulated. By comparison, EPA and DHA ethyl esters did not significantly affect any of the obesity parameters (body weight gain,

abdominal fat or hepatic steatosis) or mRNA-expression of adiponectin. It did, however, suppress the expression of pro-inflammatory genes and improved glucose tolerance, although not to the same extent as the wax ester supplement. Based on these results we concluded that the active component of Calanus oil is confined to its main lipid constituent, namely the wax ester. Wax ester had a more clear anti-obesity effect compared to EPA and DHA ethyl esters, whereas their anti-inflammatory effects were comparable.

Paper III

In this study we tested whether Calanus oil was able to attenuate angiotensin II (Ang II) - induced changes in blood pressure and cardiac remodeling in diet-induced obese mice. Thus, C57BL/6J mice were initially subjected to 8 weeks of HFD with or without 2% Calanus oil. Thereafter, animals within each group were randomized for the administration of either Ang II (1 μg/kg/min) or saline for another two weeks. Ang II caused a marked elevation in blood pressure in mice receiving non-supplemented HFD, while this response was clearly attenuated in mice receiving Calanus oil supplementation. Ang II also caused a marked decline in body and organ weights in mice receiving non-supplemented HFD, whereas this effect was less prominent in mice receiving Calanus oil supplementation. Infusion of Ang II produced cardiac hypertrophy and up-regulation of marker genes of both hypertrophy *(ANF, β-MHC)* and fibrosis *(Timp1 and Fn-1).* This response was however not affected by dietary Calanus oil. The mRNA level of fibrotic genes *(Col-α1 and Col III-α1, Fn-1)* and inflammatory genes *(TNFα and IL-6)* were also up-regulated in the aorta following Ang II infusion, while dietary Calanus oil appeared to block the inflammatory response. Interestingly, Calanus oil appeared to have a protective effect as fewer mice in the Calanus oil supplimentation group were removed due to death/sacrifice than the HFD group receiving Ang II and no oil supplementation. Finally, we demonstrated that Calanus oil led to a robust increase in cardiac protein O-GlcNAcylation, probably a protective adaptation which, in combination with the anti-inflammatory effect of Calanus oil, mitigated the adverse effects of Ang II on the cardiovascular system.

General Discussion

In this doctoral project we have shown that dietary supplementation with Calanus oil during high-fat feeding in mice was able to significantly reduce abdominal as well as ectopic fat deposition, which otherwise occurred with non-supplemented high-fat feeding. At the same time, obesity-induced low-grade inflammation in adipose tissue, as well as glucose intolerance, were attenuated. The same beneficial results were obtained when the diet was supplemented with Calanus oil-derived wax ester, indicating that the active component in Calanus oil is confined to its lipid constituent. Finally we found that dietary supplementation with Calanus oil was able to attenuate hypertension induced by Ang II infusion, as well as the accompanying condition of cachexia.

Anti-obesity action of Calanus oil

Beneficial health effects of marine oils have traditionally been ascribed to their content of n–3 PUFAs, particularly EPA and DHA, and many studies have shown that n–3 PUFAs can counteract obesity-related metabolic disturbances [93, 160, 161].Decreased energy intake [93] and suppression of lipogenesis [162, 163] are the two proposed mechanisms for the anti-obesity effect of n-3 PUFA. Calculations (based on food intake measurements) showed, however, that the anti-obesity effect of Calanus oil could not be explained in terms of reduced energy intake. Thus, it is more likely that an imbalance between fat deposition and fat mobilization can explain the reduced abdominal fat depots in response to intake of Calanus oil. In line with this notion, it has been reported that isolated adipocytes from mice given a high fat diet supplied with EPA express lower levels of glycerol-3-phosphate dehydrogenase, which is a key regulatory enzyme in the process of lipogenesis [164], and it has also been shown that administration of EPA suppresses hepatic lipogenesis [162]. Hence, it is possible that the active component of Calanus oil leads to activation of lipolysis in perirenal fat which overrides lipogenesis, thereby explaining the reduction in size of this particular fat depot. It has also been reported that astaxanthin, a strong antioxidant found in Calanus oil, can decrease the amount of abdominal fat in diet-induced obese mice [165], but in our hands this was not the case, since purified wax ester from Calanus oil (containing no astaxanthin) was able to do the job.

Finally, it should be noted that supplementing the high-fat diet with purified ethyl esters of EPA and DHA, matching the total amount of n-3 PUFA in the 1.5% Calanus oil-supplemented diet did not provide a clear anti-obesity effect, neither in the form of body weight reduction nor in reductions of abdominal fat mass or hepatic TAG content. Probably, the anti-obesity action of Calanus oil (compared to the purified ethyl esters) depends on its content of other omega-3 fatty acids and/or mono-unsaturated fatty acids (gondoic acid and cetoleic acid).

Anti-inflammatory- and insulin-sensitizing action of Calanus oil

Calanus oil (as well as wax esters derived from the oil) attenuated the inflammatory response in abdominal adipose tissue, which was accompanied by reduced adipocyte size, as well as reduced inflammatory gene expression and macrophage infiltration in abdominal fat tissue. It is well documented that there is a strong correlation between adipose cell enlargement and macrophage (M1) infiltration in the adipose tissue [99, 166]. The most accepted theory behind this observation is that expansion of adipocytes leads to local hypoxia and activation of hypoxia-inducible factor 1-alpha (HIF1α), which in turn leads to up-regulation of pro-inflammatory genes [6, 22].

In line with the well-established link between low-grade inflammation in adipose tissue and insulin resistance [99, 167], the present study showed that the reduced inflammatory state after WE supplementation was accompanied by reduced circulating glucose and insulin concentrations, as well as improved glucose tolerance. The inflammatory state was also reduced in mice receiving EPA/DHA-supplemented diet, whereas plasma glucose, glucose tolerance, and insulin values were only modestly affected. The explanation for this finding is not clear, but the markedly lower expression of the insulin-sensitizing hormone adiponectin in adipose tissue of the EPA/DHA group might be one explanatory factor.

Numerous studies show that dietary PUFAs appear to exert preventive effects on the development of insulin resistance and diabetes [168, 169], specifically when compared with saturated fatty acids that promote diabetes development [170, 171]. High n-3 PUFA content diet results in insulin sensitization due to enhanced stimulation of GPR120 and anti-inflammatory effects [101]. It has been suggested that n-3 PUFA might interfere with insulin secretion, which leads to a decrease in circulating insulin levels and a concomitant rise in blood glucose [172].

Antihypertensive and anti-cachexic action of Calanus oil

Administration of Ang II leads to elevated blood pressure, as well as cardiac remodeling. In high-fat fed mice we observed, however, that dietary supplementation with Calanus oil prevented the Ang II-induced rise in blood pressure. Ang II-induced hypertension, as reflected by increased heart weight and increased mRNA expression of hypertropic *(ANP, BNP, β-MHC)* and fibrotic genes *(Col I-α1 and Col III–α1, TIMP1 and Fn-1)* were not influenced by Calanus oil supplementation, and therefore the anti-hypertensive action of Calanus oil could be related to events at the vascular bed. Of interest, we observed that Calanus oil blunted the Ang II-induced increase in *Col I-α1* and *Col III–α1* mRNA expression in aorta, and it also effectively prevented the increase in *TNFα* and *IL-6* expression in this tissue. Thus, it appears that dietary Calanus oil prevented the Ang II-induced rise in blood pressure by reducing the inflammatory response in the vessel wall.

Another striking observation in the present study was that acute treatment of diet-induced obese mice with Ang II led to a marked decrease in body mass in comparison to saline-treated mice and, more importantly, that this effect was generally blunted in mice receiving dietary Calanus oil supplementation. Several mechanisms have been implicated for the cachexic action of Ang II. Based on studies in rats Brink et al. [173] suggested already in 1996 that Ang II infusion produces weight loss through a pressor-independent mechanism that includes a marked anorexigenic effect. Cassis et al. [174] reported that low levels of Ang II infusion regulate body weight through mechanisms related to increased peripheral metabolism (reflected as increased surface temperature), while others have reported an increase in mitochondrial uncoupling protein 2 (UCP2) expression in skeletal muscle after Ang II [175].

In an attempt to further uncover underlying causes for the response to Ang II, as well as the beneficial effect of Calanus oil, we examined cardiac tissue for general changes in protein O-GlcNAcylation. Somewhat surprisingly, we found that dietary supplementation with Calanus oil led to a general increase in protein O-GlcNAcylation in heart tissue, but realized that this may be viewed as a cardioprotective process, since it mitigated many of the adverse effects of Ang II on survival, changes in tissue mass, and the increase in blood pressure that was observed in the mice which did not receive Calanus oil. The exact mechanism of the cardioprotection afforded by increased O-GlcNAcylation is currently unknown; however,

recent studies have suggested that it may reduce or mitigate the effects of ER stress and prevent further cell damage and apoptosis [71]. Furthermore, studies on isolated cardiac myocytes have indicated that O-GlcNAcylation can limit the development of cardiac hypertrophy [72], but this effect was not observed in our model of Ang II-treated obese mice, whether they received Calanus oil or not.

Concluding Remarks

In this doctoral project we have shown that dietary supplementation with Calanus oil during high-fat feeding in mice was able to significantly reduce abdominal as well as ectopic fat deposition. The treatment significantly reduced the obesity-related low-grade inflammation in adipose tissue, while at the same time improving glucose tolerance. Collectively, these findings support the notion that low-grade inflammation in adipose tissue is the link between obesity and insulin resistance, and that reduction of visceral fat mass by Calanus oil supplementation is an obvious possibility for targeting the inflammatory network. Finally, dietary Calanus oil can antagonize Ang II-induced hypertension and cachexia, an effect that most likely should be ascribed to the anti-inflammatory action of the oil.

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Appendix

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

DESCRIPTION

Diet Induced Obesity (DIO) Rodent Diet with 45% Energy From Fat, Dyed Red is a Purified Diet based on AIN-76A Semi-Purified Diet, Rat or Mouse 5800-B. See Van Heek et al., J. Clin. Invest. 99:385-390, 1997, for initial use of this formula. Originally manufactured as "D12451".

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

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

**Other Forms Available By Request*

INGREDIENTS

Casein - Vitamin Tested	23.3060
Lard	20.6840
Sucrose	20.0920
Maltodextrin	11.6530
Dextrin	8.4830
Powdered Cellulose	5.8270
Soybean Oil	2.9130
Potassium Citrate, Tribasic Monohydrate	1.9230
Calcium Phosphate	1.5150
DIO Mineral Mix	1.1650
AIN-76A Vitamin Mix	1.1650
Calcium Carbonate	0.6410
L-Cystine	0.3500
Choline Bitartrate	0.2330
FD&C Red 40 Lake	0.0500

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

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

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

FEEDING DIRECTIONS

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

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

NUTRITIONAL PROFILE [1]

Protein, %	***20.8***
Arginine, %	0.81
Histidine, %	0.60
Isoleucine, %	1.11
Leucine, %	2.02
Lysine, %	1.69
Methionine, %	0.60
Cystine, %	0.44
Phenylalanine, %	1.11
Tyrosine, %	1.18
Threonine, %	0.90
Tryptophan, %	0.26
Valine, %	1.33
Alanine, %	0.64
Aspartic Acid, %	1.50
Glutamic Acid, %	4.76
Glycine, %	0.45
Proline, %	2.75
Serine, %	1.29
Taurine, %	0.00
Fat, %	***23.6***
Cholesterol, ppm	197
Linoleic Acid, %	3.48
Linolenic Acid, %	0.32
Arachidonic Acid, %	0.04
Omega-3 Fatty Acids, %	0.32
Total Saturated Fatty Acids, %	9.05
Total Monounsaturated Fatty Acids, %	9.32
Fiber (max), %	***5.8***
Carbohydrates, %	***41.2***
Energy (kcal/g) [2]	***4.60***

From:	kcal	%
Protein	0.833	18.1
Fat (ether extract)	2.124	46.1
Carbohydrates	1.648	35.8

Minerals	
Calcium, %	0.71
Phosphorus, %	0.53
Phosphorus (available), %	0.53
Potassium, %	0.70
Magnesium, %	0.06
Sodium, %	0.14
Chloride, %	0.24
Fluorine, ppm	1.1
Iron, ppm	58
Zinc, ppm	41
Manganese, ppm	68
Copper, ppm	7.0
Cobalt, ppm	0.0
Iodine, ppm	0.24
Chromium (added), ppm	2.3
Molybdenum, ppm	1.90
Selenium, ppm	0.27

Vitamins	
Vitamin A, IU/g	4.7
Vitamin D-3 (added), IU/g	1.2
Vitamin E, IU/kg	60.6
Vitamin K, ppm	0.59
Thiamin Hydrochloride, ppm	7.1
Riboflavin, ppm	7.8
Niacin, ppm	35
Pantothenic Acid, ppm	19
Folic Acid, ppm	2.5
Pyridoxine, ppm	6.8
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	16
Choline Chloride, ppm	1,165
Ascorbic Acid, ppm	0.0

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly. Nutrients expressed as percent of ration on an As Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

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TestDiet
www.testdiet.com

3/25/2014

DIO Rodent Purified Diet w/10% Energy From Fat - Yellow 58Y2

DESCRIPTION

Diet Induced Obesity Rodent Purified Diet with 10% Energy From Fat, Dyed Yellow is based on AIN-76A Semi-Purified Diet, Rat or Mouse 5800-B. See Van Heek et al., J. Clin. Invest. 99:385-390, 1997, for initial use of lower-fat versions of this formula. Originally manufactured as "D12450B".

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

Product Forms Available*	***Catalog #***
1/2" Pellet	58124
1/2" Pellet, Irradiated	56834
Meal	1810727
Meal, Irradiated	1810728

**Other Forms Available On Re*

INGREDIENTS (%)

Sucrose	33.1290
Dextrin	29.8560
Casein - Vitamin Free	18.9560
Powdered Cellulose	4.7390
Maltodextrin	3.3170
Soybean Oil	2.3700
Lard	1.8960
Potassium Citrate, Tribasic Monohydrate	1.5640
Dicalcium Phosphate	1.2320
DIO Mineral Mix	0.9480
AIN-76A Vitamin Mix	0.9480
Calcium Carbonate	0.5210
L-Cystine	0.2840
Choline Bitartrate	0.1900
Yellow Dye	0.0500

NUTRITIONAL PROFILE [1]

Protein, %	***17.3***
Arginine, %	0.66
Histidine, %	0.49
Isoleucine, %	0.91
Leucine, %	1.64
Lysine, %	1.38
Methionine, %	0.49
Cystine, %	0.35
Phenylalanine, %	0.91
Tyrosine, %	0.96
Threonine, %	0.73
Tryptophan, %	0.21
Valine, %	1.08
Alanine, %	0.52
Aspartic Acid, %	1.22
Glutamic Acid, %	3.87
Glycine, %	0.37
Proline, %	2.23
Serine, %	1.05
Taurine, %	0.00
Fat, %	***4.3***
Cholesterol, ppm	18
Linoleic Acid, %	1.39
Linolenic Acid, %	0.19
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.19
Total Saturated Fatty A	1.14
Total Monounsaturated Fatty Acids, %	1.30
Polyunsaturated Fatty Acids, %	1.59
Fiber (max), %	***4.7***
Carbohydrates, %	***67.4***
Energy (kcal/g) [2]	***3.78***

From:	kcal	%
Protein	0.692	18.3
Fat (ether extract)	0.384	10.2
Carbohydrates	2.697	71.5

Minerals	
Calcium, %	0.57
Phosphorus, %	0.43
Phosphorus (available), %	0.43
Potassium, %	0.57
Magnesium, %	0.05
Sodium, %	0.12
Chloride, %	0.21
Fluorine, ppm	0.9
Iron, ppm	44
Zinc, ppm	34
Manganese, ppm	55
Copper, ppm	5.7
Cobalt, ppm	0.0
Iodine, ppm	0.20
Chromium, ppm	1.9
Molybdenum, ppm	1.55
Selenium, ppm	0.15

Vitamins	
Vitamin A, IU/g	3.8
Vitamin D-3 (added), IU/g	0.9
Vitamin E, IU/kg	49.3
Vitamin K (as menadione), ppm	0.48
Thiamin Hydrochloride, ppm	5.7
Riboflavin, ppm	5.7
Niacin, ppm	28
Pantothenic Acid, ppm	14
Folic Acid, ppm	1.9
Pyridoxine, ppm	5.5
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	9
Choline Chloride, ppm	950
Ascorbic Acid, ppm	0.0

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

FEEDING DIRECTIONS

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

CAUTION:
Perishable - store properly upon receipt.
For laboratory animal use only; NOT for human consumption.

6/28/2007

ISO 9001:2000 CERTIFIED

TestDiet
www.testdiet.com

Paper I

Paper II

Paper III