The Effect of Krill Oil and Krill Hydrolysate Protein on Fat Storage and Metabolic health in Male Wistar Rats

By

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Abbreviations

BMI	Body mass index
CHD	Coronary heart disease
CVD	Cardiovascular disease
DMT2	Diabetes mellitus type 2
EGIR	European Group for the study of Insulin Resistance
FA	Fatty acids
FFAs	Free fatty acids
GLUT4	Glucose transporter type 4
IR	Insulin resistance
IGT	Impaired glucose tolerance
IDF	International Diabetes Federation
HDL	High density lipoprotein
HF	High fat
HGP	Hepatic glucose production
IHTG	Intrahepatic triglyceride
KO	Krill oil
KH	Krill hydrolysate
PRI	Pristine (KO+KH)
LDL	Low density lipoprotein
LF	Low fat
MetS	Metabolic syndrome
MUFA	Monounsaturated fatty acid
NCEP-ATP III	National Cholesterol Education Program, Adult Treatment Panel III
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acids
T2DM	Type 2 Diabetes Mellitus
TAG	Triacylglycerol
WHO	World Health Organization

ABSTRACT

Background: Metabolic syndrome is high and rising across the world. To combat this, lifestyle changes have been deemed to be crucial for managing metabolic syndrome and its comorbidities. Physical activity and dietary changes are included in this lifestyle modification. Dietary interventions with marine-derived oils have been suggested to be beneficial for metabolic health by exerting anti-inflammatory and hypertriglyceridemic effects. This is due to their abundant content of bioactive lipids, docosahexaenoic acid (DHA) and eicosatetraenoic acid (EPA), and possibly also bioactive protein/peptides
Therefore, more sources of marine oils is desired. Krill is such source. However, the impact of these respective components of krill (oil and protein/peptides), and whether these compounds have additive or synergistic effect, need better documentation.

Method: Male Wistar rats (n=40) were randomly divided into 5 groups fed a high-fat (HF) Western diet (n=8, control group), krill oil (KO) (n=8), krill hydrolysate (KH) (n=8), or the combination of these (krill oil + krill hydrolysate, called "pristine" (n=8), for 8 weeks. All groups consumed the same amount of total energy. A low fat (LF) was also given for comparison to the high-fat diet (n=8). The intervention groups of krill oil, krill hydrolysate and pristine were given together with the high-fat Western diet as the background diet. Body-, organ-, and fat tissue weights were measured, as well as plasma glucose and standard lipids (e.g. triglyceride and cholesterol), fatty acids composition in plasma and liver were also measured.

Results: The individual diets of krill oil and krill hydrolysate did not have any marked effect on body- and organ weight and plasma glucose lipids of the rats. However, in KO+KH fed rats, there were significant increases in body- and organ weight was significantly high when compared to HF-diet fed rats and the rest of the individual diets. No significant differences were observed in plasma glucose and standard lipids between any of the intervention groups. Plasma and liver concentrations of EPA and DHA were however significantly higher in the rats that consumed krill oil and pristine, confirming that the krill-based supplements increased the organismal levels of these bioactive lipids.

Conclusion: No added beneficial effect on metabolic health was observed when supplementing male Wistar rats with krill oil or krill hydrolysate together with a high-fat dietary background. Rather, the combination of krill oil and krill hydrolysate had a weight- and fat mass-increasing effect despite similar energy intake across the groups.

INTRODUCTION

METABOLIC SYNDROME DEFINITION

Metabolic syndrome (MetS) or insulin resistance syndrome is a cluster of ailments that is associated with increased risk of diabetes and atherosclerotic disease (1,2). The factors used to define the syndrome include, increased waist-to-hip ratio or waist circumference, hyperglycemia, or use of glucose-lowering drugs, increased triacylglycerols (TAG), decreased high-density lipoprotein cholesterol (HDL-C), high blood pressure (hypertension) and microalbuminuria (3). Originally the concept was developed by Gerald Reaven in 1988, at the time referred to as syndrome X (4). Since then, the term metabolic syndrome has been institutionalized by many international organizations and expert groups such as World Health Organizations (WHO), the European Group for the study of Insulin Resistance (EGIR) and the National Cholesterol Education Program, Adult Treatment Panel III (NCEP-ATP III) (1). These organizations have slightly different definitions of metabolic syndrome. WHO defines it as having at least two of the components (5).

Table 1: Definitions of metabolic syndrome as identified by various organizations.

WHO (1998)(6)	**EGIR (1999)**(7)	**NCEP-ATP III (2001)**(8)	**IDF (2006)**(9)
Insulin resistance (T2DM or IFG or IGT) and two of the following: 1. WHR >0.9 (men) and 0.85 (women) or BMI ≥30 kg/ m^2 2. Plasma TAG ≥ 1.17 mmol/l (150 mg/dl) 3. HDL-C <0.9 mmol/l (35 mg/dl) (men) and <1,0 mmol/l (women) 4. BP ≥ 140/90 mmHG	25 % among non-diabetic population with highest fasting hyperinsulinemia or insulin resistance and two of the following criteria: 1. FPG ≥ 6 > 1 mmol/l 2. WC ≥ 94 cm (male) or ≥80 cm (women) 3. TAG > 2.0 mmol/l or HDL-C < 1.0 mmol/l (39 mg/dl) (women)	Any three or more of the following criteria: 1. FPG ≥ 6.1 mmol/l (110 mg/dl) 2. WC > 102 cm (men) and > 88 cm (women) 3. Plasma TAG ≥ 1.7 mmol/l 4. HDL-C <1.0 mmol/l (<40 mg/dl) (male) and <1.3 mmol/l (<50 mg/dl) (women)	WC > 95 cm (men) or > 80 cm (women) along with the presence of two or more of the following: 1. FPG ≥ 5.6 mmol/L (100 mg/dl) 2. TAG ≥ 1.7 mmol/l (150 mg/dl)

	< 35 mg/dl (men) 4. BP≥ 140/90 mm Hg or on antihypertensive therapy.	5. BP ≥ 130/85 mmHG	3. HDL-C < 1.03 mmol/l (40 mg/dl) (men) <1.29 mmol/l (50 mg/dl) (women) 4. BP ≥130/85 mm HG or on antihypertensive therapy.

T2DM, type 2 diabetes mellitus; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; WHR, waist-hip-ratio; BP, blood pressure; TAG, triacylglycerols; HDL-C, high-density lipoprotein cholesterol; WC, waist circumference; WHO, World Health Organization; EGIR, European Group for the study of Insulin Resistance; NCEP-ATP III, National Cholesterol Education Program, Adult Treatment Panel III; IDF, International Diabetes Federation.

PREVALENCE OF METABOLIC SYNDROME

The prevalence of metabolic syndrome varies and is dependent on the criteria used to determine inclusion and the characteristics of the population studied (race, ethnicity, age, sex). Nevertheless, it has become apparent that despite which definition or criteria used, the prevalence of metabolic syndrome is high and increasing in all western communities. The incidence of the syndrome is rising in accordance with the increase in the number of people with overweight and obesity (2). Incidence comorbidities affiliated with metabolic syndrome also varies by ethnicity. Especially, obesity-related comorbidities (type 2 diabetes mellitus (T2DM), hypertension, dyslipidemia) appears more frequently at a lower body mass index in Asians than in white Caucasians (10) By using NCEP-ATP III criteria, a large study conducted on the US population estimated the age-adjusted and unadjusted prevalence of metabolic syndrome to be 23.7% and 21.8%, respectively (11). It should be noted that the definition criteria used by WHO and EGIR is more restrictive as opposed to the commonly used criteria of the NCEP-ATP III and the International Diabetes Federation (IDF) (12).

According to the National Health and Nutrition Examination Survey (NHANES) analysis of 1988 - 2010, the average body mass index (BMI) in both men and women in USA increased by 0.37% per year. Consequently, average waist circumference increased by 0.37% and 0.27%

per year in women and men, respectively (13). According to National Diabetes Statistic reports published by Center for disease control and prevention (CDC) in 2017 (14), approximately 30.2 million adults aged 18 years or older or 12.2% of USA adults had T2DM, of which 7.2 million of these people (23.8%) were not even aware of or did report having diabetes. Incidence of T2DM increased with ages, reaching up to 25.2% among US seniors (65 years and older). The prevalence of prediabetes and metabolic syndrome was approximately 3 times more than those who had T2DM, and so about one third of US adults are estimated to have metabolic syndrome (14).

Furthermore, in China, in the years between 1992 and 2002, the prevalence of overweight and obesity escalated from 14.6 to 21.8% (5,15). However, using the Chinese obesity definition, with lower BMI cutoff, the increase went from 20 – 29.9%. Incidence of metabolic syndrome was higher in urban areas with an increase from 8 – 10.6% and 4.9 – 5.3 in rural areas (5,15).

Globally, a survey done in 195 countries in 2015 showed that 604 million adults and 108 million children were obese. The prevalence of obesity has doubled in 73 countries and increased in most other countries since 1980. What was even more worrying was that the rate of increase was higher for childhood obesity (5). According to the survey, obesity no longer has the status of a disease for the wealthy, as the disease was most prevalent in young men (25-29 years) in countries with low socio-economic index (SDI). The prevalence has in the last three decades gone from 1.1% in 1980 to 3.85% in 2015. Furthermore, the survey reported between 1990 and 2015, global death rate related to high BMI increased by 28.3% (16). Global prevalence of diabetes was 8.8% (415 million) in 2015 according to IDF diabetes atlas (17) and was then expected to rise to 10.4% (642 million) by the year 2040 (5).

Complications/morbidity related to metabolic syndrome

The need to accurately define MetS arise from the need to detect accurately individuals at high risk for cardiovascular disease (CVD) and T2DM. All the components in the various MetS definitions are involved in giving risk to CVD and T2DM. In particular, the three factors of atherogenic dyslipidemia (increased LDL, decreased HDL-C and increased TAG in the blood) are independently associated with cardiovascular risk, whereas the risk of developing T2DM increases significantly with IR (2). Several studies have shown central obesity to be associated with an increased risk of CVD and T2DM. It has been confirmed by several epidemiological

studies that individuals with metabolic syndrome have increased risk to develop CVD despite the diagnostic criteria used (2).

Pathophysiology/ mechanism of metabolic syndrome

The exact etiology of metabolic syndrome is still not quite understood and therefore remain to be fully elucidated. There are continuous discussions about whether the different elements of MetS represent distinct pathologies or manifestations of a common pathogenic mechanism (18). In addition to genetic and epigenetic factors, lifestyle, and environmental factors such as excessive intake of high-calorie food combined with lack of physical activity are major contributors (18). Given that visceral adiposity has been shown to be a primary trigger for most pathways involved in metabolic syndrome, the causative role can be attributed to high calorie intake with low energy expenditure (18,19).

Among the proposed mechanisms, insulin resistance seems to be the most widely accepted underlying cause (20). Although the origins of insulin resistance are debated, contributing factors are likely to be adipose tissue dysfunction, chronic inflammation, oxidative stress (21).

Obesity – worldwide growing health problem

Obesity described as increased adipose tissue mass is one of the main driving forces in insulin resistance and pathogenesis of T2DM and metabolic syndrome (22). The incidence of obesity is rising dramatically in almost all societies in the world, accompanied by important pathological consequences such as T2DM and CVD. WHO estimates that the global prevalence of obesity has almost tripled between 1975 and 2016 (23). Just in 2016, over 1.9 billion adults, (18 years and above) comprising 39% of the world adults were reported to be overweight, and at least 650 million of these had obesity (23). As a result, death related to overweight, and obesity has become more frequent globally than death caused by being underweight (23).

Obesity – definition

WHO defines obesity as “abnormal or excessive fat accumulation that may impair health”(23). The high fat storage is due to the body being overloaded with energy through high-calorie food exceeding what the body needs to carry out necessary energy-intensive processes. Thus, the body will store excess energy from the food in the adipose tissue (24). High fat content is usually seen in the intra-abdominal area, also known as visceral fat, and in subcutaneous

adipose tissue especially on the abdomen, thighs, buttocks, and hip areas (24). BMI is the measure commonly used to classify obesity in the population. BMI is calculated by dividing body weight (kg) by the square of height (m^2). WHO defines overweight as BMI ≥ 25 kg/m^2 and obesity as BMI ≥ 30 kg/m^2 for adults (23). Obesity assessment using BMI has its restrictions, in part because the measure does not perfectly correspond to body fat nor its distribution. Therefore, additional parameters used to classify obesity include waist circumference (cm) and waist-hip ratio (WHR). These are measures of central obesity and are used as surrogate estimate of visceral adiposity, which is linked to insulin resistance, dyslipidemia, and increased risk of CVD (25). According to WHO, central obesity is defined as waist circumference above 102 cm in men and above 88 cm in women and WHR of 0.90 and above for men and 0.85 and above for women (26).

Obesity is a condition that is characterized with chronic low-grade inflammation in metabolic tissues that are involved in energy homeostasis and is often denoted as metabolic inflammation or "metaflammation" (27). This gradual state of inflammation is often caused by a number of events. Exposure to free fatty acids (FFAs) is one of them. When exposed to FFA, the inflammatory signaling pathway gets activated. In addition, immune cell infiltration and shift in inflammatory cell population contribute to inflammation in metabolic tissues. In the absence of obesity, the adipose tissue is effective at storing FFA. However, an obese state, the adipose tissue can no longer store FFA effectively due to its storage capacity being exceeded. Thus, FFA "spill over" and build up in metabolic tissues such as the skeletal muscle, liver, and pancreas causing lipotoxicity. Consequently, lipotoxicity in these tissues can activate in inflammatory pathways and therefore impair normal signaling in these tissues and hence cellular dysfunction. Cellular dysfunction in skeletal and liver cells can consequently affect the insulin-signaling pathway and this can lead to insulin resistance and T2DM (28).

Body fat distribution

Distribution of fat in the body variates in both lean and obese adults and is affected by a number of factors. Some of the factors include smoking, alcohol consumption and time of onset of childhood obesity. Additionally, genetic susceptibility appears to play a role in regional fat gain and loss. A predominant fat distribution in the upper body is usually linked to increased visceral fat (29). Imaging studies have shown that visceral fat is often accompanied by high liver fat accumulation and non-alcoholic fatty liver disease (NAFLD) (30).

Fatty liver disease

In addition to being the most important detoxification organ in the body, the liver also plays a vital role in the regulation of lipogenesis, gluconeogenesis, and metabolism of cholesterol. Furthermore, the liver is responsible for metabolic homeostasis (28). However, the liver can be impacted negatively by lipotoxicity and associated metabolic inflammation induced by obesity (28,31). Obesity is strongly associated with the liver disorder non-alcoholic fatty liver disease (NAFLD), characterized by increased buildup of TAG (steatosis) in hepatocytes with or without inflammation and fibrosis (i.e steatohepatitis and cirrhosis) (32). NAFLD has become an important health issue in modern society, with a high occurrence and contribution to severe liver illness and its linkage to serious CV disorders, as well as T2DM, metabolic syndrome and CHD. NAFLD is therefore considered the hepatic manifestation of the metabolic syndrome (33).

Adipose tissue and obesity

Adipose tissues are important energy storing organs that play a major role in metabolic homeostasis and fatty acid metabolism. This loose connective tissue comprises of adipocytes, preadipocytes, vascular endothelial cells, fibroblasts, and several types of immune cells including adipose tissue macrophages (34). In light of the global obesity epidemic in recent years, growing interest has been shown towards adipose tissue biology. Following the discovery of leptin in 1994, the adipose tissue has gained status as an active endocrine organ that secretes numerous hormones including adiponectin and other adipokines including various cytokines (35).

Adipose tissue is divided into 3 main categories: white adipose tissue (WAT), brown adipose tissue (BAT) and beige adipose tissue (22). The different adipose tissue depots are distributed throughout the body and has been shown to have different functional properties. Anatomically, WAT are in visceral and subcutaneous depots. The function of WAT is to store excess energy in the form of TAG and release fatty acids during periods of fasting. BAT have more mitochondria and generate thermogenesis via uncoupling of oxidative phosphorylation which is mediated through uncoupling protein-1 (UCP-1), an inner mitochondrial membrane integral protein. While BAT depots are usually seen around the heart and large vessels in toddlers and infants, there is little left in adults and WAT dominates (36).

Moreover, the adipose tissue poses different risks for metabolic diseases depending on where the body fat is located. Additionally, the distribution of subcutaneous and visceral adipose tissue variates among individuals and depends on factors such as age, sex, nutrition, and energy balance of the individual adipose tissues (37). The localization of fat is of more importance in terms of risk of metabolic diseases than the total mass buildup of visceral intra-abdominal WAT, better known as central obesity, is associated with insulin resistance and increased risk of metabolic disorder while buildup of subcutaneous WAT, i.e. in the lower body (buttocks and thighs), have no negative impact and may also be protective against metabolic syndrome (22,37).

The Body fat increase that is seen in obesity involves an expansion in the size (hypertrophy) and/or quantity (hyperplasia) of adipocytes (38). People with metabolically healthy obesity have smaller adipocytes in comparison to those who have metabolically unhealthy obesity, linking adipocytes enlargement to development of metabolic disorders (35,39).

Increased adipocyte size affects metabolic and hemodynamic processes and the production and release of adipokines. Changes in the release of adipokines, such as inflammatory cytokines, can promote insulin resistance and atherosclerosis (39). Therefore, a proper functioning adipose tissue with ability to neutralize and store surplus nutrient, is essential to protect the body from peripheral insulin resistance (37).

INSULIN RESISTANCE

Systemic insulin resistance is characterized by fasting hyperinsulinemia and/or hyperglycemia in the presence of normal or elevated fasting insulin. To understand insulin resistance, it is important to understand the basis of insulin action and its signaling cascades. Under normal healthy circumstances, an increase in blood glucose during feeding stimulates the release of insulin from pancreatic β cells. Insulin, along with glucose, stimulates the uptake of glucose from the circulation into cells for glycolysis or storage as glycogen and lipid in the liver, muscle or adipose tissue, and suppresses hepatic gluconeogenesis. All these physiological processes collaborate to lower blood glucose after a meal to the normal basal level range. Glucose transporter type 4 (GLUT4) is one of the most important glucose transporters, as the primary insulin-responsive member of the GLUT family. This glucose transporter is expressed mainly in the muscle and adipose tissue. During insulin stimulation, GLUT4 is mobilized from the

cytosol to the cell membrane to transport glucose from outside of the cell to inside. Thus, this is the rate-limiting step in glucose uptake and muscle glycogen synthesis. In addition to regulating glucose metabolism, insulin modulates lipid metabolism. Lipogenesis increases in response to insulin, while lipolysis is suppressed (21).

However, in an abnormal or insulin-resistant condition, loss of initial insulin secretion (first phase) in response to a glucose load, leads to postprandial hyperglycemia. To counteract, an excessive second phase response of insulin causes chronic hyperinsulinemia. Insulin-responsive tissues is unable to sensitize or respond effectively to insulin. Insulin-mediated glucose uptake, glycolysis and glycogen synthesis are all impaired (21).

The effect of insulin resistance on target tissues

Adipose tissue:

In insulin resistance, the reaction of insulin-responsive tissues to insulin is very inefficient. Development of insulin resistance in adipose tissue, impairs insulin-mediated inhibition of lipolysis. This then leads to an increase in circulating FFAs, which further inhibits the antilipolytic effect of insulin. The resulting increase in FFAs in turn exacerbates insulin resistance by causing changes in the insulin signal cascade in various organs, therefore creating a vicious cycle (18).

Liver and muscle

In muscles, FFAs inhibit protein kinase activation, which leads to reduced GLUT-4 translocation to the cell membrane thus decreasing glucose uptake. In the liver however, FFAs promote gluconeogenesis and lipogenesis. A hyperinsulinemic state is reached as a net result to preserve euglyclemia. This compensation may eventually fail, with insufficient compensatory insulin production and potentially also an absolute reduction in insulin secretion, which is further worsened by the lipotoxic effect of FFAs on pancreatic β-cells (18,19).

Methods used to measure insulin sensitivity

The gold standard for assessing insulin resistance in human is the euglycemic-hyperinsulinemic clamp. The procedure was first developed by Andres and DeFronzo in 1979 and is widely accepted as reference standard for directly assessing metabolic insulin sensitivity in humans. The procedure involves high dose intravenous insulin infusion after an overnight fast. The infusion is administered at a constant rate that ranges from 5-120 mU/m^2/min (dose per body surface area per minute). This constant infusion causes hyperinsulinemia, a new steady-state insulin level that is above the fasting level. Consequently, glucose uptake in muscle and adipose tissue rises while glucose production in the liver is suppressed (40). Steady-state condition for plasma insulin, blood glucose and glucose infusion rate (GIR) are typically achieved after several hours of constant insulin infusion. Provided that the hyperinsulinemic condition is enough to entirely hinder liver glucose production, and since there is no net change in blood glucose concentrations under steady state clamping conditions, the GIR must be equivalent to the glucose disposition rate (M). This concept is limited to use in research settings and is difficult to apply for larger populations studies and in clinical practice as it is time-consuming and expensive. For this reason, most such studies use a measure of fasting as a surrogate for insulin resistance (40).

The homeostasis model assessment of insulin resistance (HOMA-IR) is a model that is used to provide an estimate of insulin sensitivity and β-cell function from fasting plasma insulin and glucose concentrations. The ratio between glucose and insulin in the basal state reflects the balance between hepatic glucose production and insulin secretion, which is maintained by a feedback loop between the liver and β-cells (41). HOMA-IR is defined by the product of the fasting glucose and fasting insulin divided by a constant: HOMA-IR= ([fasting insulin (μU/ml)] *[fasting glucose (mmol/l)])/22.5.

LIFESTYLE INTERVENTIONS

Lifestyle changes have been deemed to be crucial for managing metabolic syndrome and its comorbidities. Average weight reduction of 7-10% in baseline body weight over a period of 6-12 months, reduction of calorie intake by 500 – 1000 calories per day and participating in physical activity for a minimum of 150 minutes per week is seen as essential strategy. Dietary changes, including a 25-35% daily reduction in fat, and reduced intake of SFA, trans FAs, cholesterol and refined carbohydrates, are all associated with regulating other MetS risk factors

like dyslipidemia, hyperglycemia and hypertension (42). Quality diets, such as Mediterranean diet, the Nordic diet and Dietary approach to Stop Hypertension (DASH) diet, have been shown to protect against Mets or to improve its indicative factors (42). There has been growing interest in the use of natural compounds to control the risk and development of MetS. Supplemental diets that provide health benefits in addition to basic nutritional value, are termed nutraceuticals and include unsaturated FAs, which generally have high concentrations of mono- and polyunsaturated fatty acids (MUFAs and PUFAs). These molecules have lipid-lowering effect and appear to do so by regulating the gene expressions mostly involved in *de novo* lipogenesis and FAs oxidation (19,42).

DIETARY FATTY ACIDS

Lipids are important macronutrients in the human diet and may exert metabolic and pharmacological effects useful for the prevention and treatment of chronic illnesses in humans. Both the quality and quantity of dietary lipids consumed have been to affect health and whole-body physiology (34). Dietary fatty acids (FAs) make up structural building blocks of cell membranes and can function as pro- and anti-inflammatory mediators, making them key elements for development, growth, and protection of human health (43).

To mitigate inflammation and other obesity-related comorbidities, proposed dietary measures include reducing the intake of saturated fatty acids (SFA) and increasing the intake of MUFAs and PUFAs (especially ensuring adequate intake of omega-3 PUFA). These dietary measures may confer atherosclerotic and CVD protection (21).

Both the quality and quantity of dietary fat have undergone major changes over the last 10 000 years. This, together with the industrial revolution has led to a change from a diet rich in omega-3 PUFA to a Western diet almost devoid omega-3 PUFA and with higher levels of trans fatty acids (TFA) and omega-6 PUFA. Consequently, this change in dietary intake has significantly increased the omega-6/omega-3 ratio from 1:1 to 20:1 (43).

Structurally, FAs are made up of hydrocarbon chain with a methyl group at one end of the molecule, referred to as the omega (ω) end and a carboxyl group at the other end (44). FAs are often characterized as short-chain (2C – 10C), medium-chain (12C-14C), long-chain (16C-18C) or very long-chain fatty acids (> 18 C) depending on the number of carbon atoms that are

present on the chain, (45). FAs are also categorized according to the number of double bonds available on the hydrocarbon chain, with saturated fatty acid (SFA) indicating no double bonds and unsaturated fatty acid indicating presence of one or more double bonds. Therefore, the FAs containing one double bond is referred to as monounsaturated fatty acid (MUFA) while the one containing more than one double bond is referred to as polyunsaturated fatty acid (PUFA) (45). Additionally, MUFAs can appear in two formations, *cis* and *trans* conformation.

Essential fatty acids

Essential fatty acids (EFAs) are necessary for many physiological systems to function properly. This subgroup of fatty acids is termed as "essential", as the human body lacks the ability to synthetize them endogenously. Therefore, they must be obtained from dietary sources to meet the biological requirement (46). These fatty acids include linoleic acid (LA) and alpha-linoleic family (ALA), parent compounds of omega-6 and omega-3 families of FAs, respectively (47).

Omega-3 FAs generally make up a small fraction of the total daily consumption of fatty acids in Western societies. The most prominent representatives of dietary omega-3 FAs are alpha-linoleic acid (C18:3n-3), Eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C20:5n-3). Plant oils such as flax and canola are enriched in omega-3 FAs known as alpha-linolenic acid (ALA), a precursor metabolite for EPA (C20:5n-3), DPA, (C22:5n-3) and DHA (C22:6n-3), although this conversion is very limited and inefficient. Rich sources of EPA and DHA are fish such as tuna, trout and salmon. Supplements of fish oil are also abundant source, as they usually contain 30%-50% omega-3 FA by weight (48).

Fish oil supplements

Dietary interventions with marine-derived oils have shown clinical benefits, particularly due to the effects of the long chain omega-3 polyunsaturated fatty acids (PUFAs) docosapentaenoic acid (DHA) and eicosapentaenoic acid (EPA). These bioactive lipids are found in marine food sources such as fish and seafood. Regular intake of fish (particularly fatty fish) in the diet has been reported to be beneficial for cardiovascular health and have shown anti-inflammatory and exert hypotriglyceridemic effect (34,39). Therefore, these FAs can help treat and prevent comorbidities in obesity, specifically by bettering individual components of the metabolic syndrome (34).

The dietary guidelines of the American Heart Association suggest that healthy individuals should consume at least two meals of fish per week which should provide an intake of approximately 400 – 500 mg EPA and DHA, while an of intake 1g EPA+DHA per day is recommended for persons diagnosed with CHD (49).

Studies have shown that omega-3 PUFA have effect in reducing subcutaneous adipose tissue in human. The mechanism behind how omega-3 PUFA reduces subcutaneous adipose tissue lies with its ability to bind and/or regulate transcriptional factors that control genes involved in preadipocyte differentiation. PUFAs and its metabolites, serve as ligands for peroxisome proliferator-activated receptors (PPAR)- gamma and delta to induce differentiation of fat cell and accelerate maturation by elevating the expression of lipoprotein lipase (LPL). Concentration differences in fatty acids are more strongly linked with abdominal subcutaneous than visceral adipose tissue (34).

Some studies have reported increase in adiponectin following dietary supplementation with omega-3 (50) and increase in leptin levels. However, one study reported non-significant increase while two other studies reported minimal changes (50). Adiponectin has modulatory effects on energy homeostasis, glucose and lipid metabolism and it also promotes fatty acid oxidation and enhancement of insulin sensitivity in the liver and within skeletal muscle (50). Low plasma levels of adiponectin are negatively associated with obesity, with lower levels correlated with increased risk of death or myocardial infarction (MI) (50). On the other hand, high leptin levels are positively correlated with fat mass and adipocyte size. Thus, increase levels of leptin serves as anti-obesity through decreased food intake and increased energy expenditure (50) This study shows that omega-3 dietary supplement increased leptin levels in obese subjects and in rats (50).

Omega-3 effect on adipose tissue inflammation

Studies have shown that omega-3 has an anti-inflammatory effect in adipose tissue (51,52). There is a correlation between obesity and inflammation (34). Omega-3 has a documented role in inhibiting nuclear transcription factor kappa B, a key transcription factor in cytokine gene expression and inflammation (34). Furthermore, n-3 PUFAs also have a documented effect in

reducing inflammatory cytokines, including interleukin (IL-1 and 6) and TNF-α which are all elevated in obesity (34).

Omega-3 fatty acids have a documented effect on reducing fat storage in adipose tissue and in improving some components of metabolic health via decreasing plasma TAG levels. There is evidence that FO supplementation decreases adiposity in animals that were fed with high fat diets even though FA uptake was stimulated due to expression of lipoprotein lipase (LpL) and of CD36, which is the major FA membrane transporter (53). Moreover, omega-3 FAs are reported to lower plasma TAG (53). Although, the lower effect is not present in typical western diet (about 130 mg/day) they manifest at "pharmacologic" doses (>3 g/day of EPA + DHA) (53). It is known that high plasma TAG concentration is a characteristic feature of the metabolic syndrome (MetS) (54), reduction in plasma TAG will thus reduce MetS phenotype. In addition, TAG lowering effect has cardiovascular benefit (53).

KRILL

The ever-increasing consumption and declining resources of fish has led to the search for new marine food sources for human consumption. Krill is one such source (55). Krill are small shrimp-like crustacean found in all oceans of the world. There are 85 krill species recorded worldwide with a size ranging from 0.01 to 2 grams and 8 mm to 6 cm in length. These species are abundant and are commonly found in open sea areas. Despite their small size, krill can appear in dense swarms on the surface of the water making them an easy target to harvest. Although they are mostly known as a food source for whales, they are also an important food source for other fish, seabirds, and sea mammals. Like other crustaceans, krill have a chitininous external skeleton, which is divided into three segments namely cephalon, thorax and abdomen, though sometimes some of the segments cannot be differentiated as the head and the thorax are fused into the cephalothorax. Krill can be distinguished from other crustaceans due to their visible outer gills, luminous organs and cephalothorax content that consist of highly proteolytic enzymes (55). Of the different krill species, the most globally abundant one in both quantity and biomass is the Antarctic krill, *Euphasia superba*. This krill species is of particular importance as it is harvested for human consumption and is the source of extracted krill oil. *Euphasia superba* is found in the cold waters of southern Antarctic areas and is 5-6 cm of length and has a lifespan of 6-7 years (56,57).

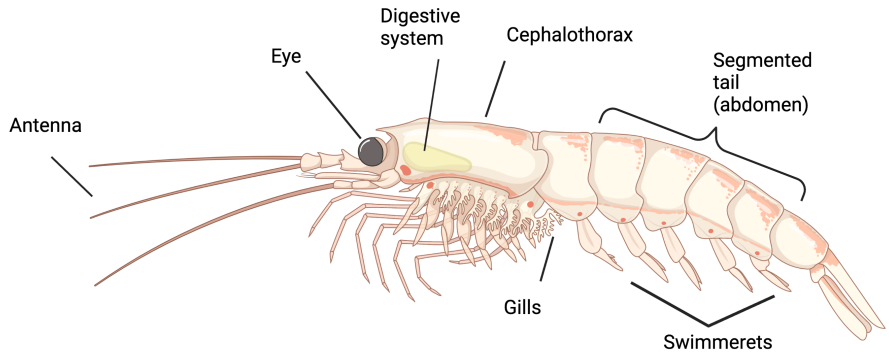

Figure. Antarctic krill, *Euphasia superba* adapted from biorender.com

NUTRIENT COMPOSITION OF KRILL

Approximate analysis of whole krill showed a range of 77.9 % - 83.1% for moisture, 0.5% - 3.6% for total lipids, 11.9% - 15.4% for crude protein, 3% for ash, and 2% for chitin and glucides (55). Thus, krill is a good source of protein, and although relatively low in fat is a source also of oil.

Krill oil has recently surfaced on the market as a new source of omega-3 PUFA. In addition, krill oil consists of antioxidant carotenoid astaxanthin, vitamin A and E, phospholipid, and various other fatty acids. This compound is endowed with powerful antioxidant properties and is responsible for the typical dark red color seen in krill oil (58). Several studies have been done in recent years, examining the role of krill as a nutraceutical for improvement of human health. This is due to the rich nutrient content profile in krill together with the large biomass available. Consumable krill products that are currently available commercially include frozen raw krill, frozen boiled krill, and peeled krill meat, as well as krill oil supplements that come as soft gels (55).

Krill oil and fish oil are both rich in omega-3 PUFA that are mostly EPA and DHA, but the composition of krill oil has one fundamental distinction from fish oil. Whilst the EPA and DHA in fish oil is found in TAG, in krill oil these FAs are incorporated into phospholipids molecules particularly phosphatidylcholine (PC) (59). More than 80% of EPA and DHA in KO are found

in the PC form (60). Moreover, it has been reported that 29.9% of the lipid content of krill comprises of phospholipid while another report suggested higher levels of 54% to 58%. This variation in krill phospholipid content could be due to differences in krill species, age, season, or harvest time (55).

Phospholipids have been proposed to facilitate a more effective transmission of long-chain PUFAs to organs such as the brain, liver and kidney, thereby increasing bioavailability. Although one study showed lower tissue deposition of krill than fish (61). Furthermore, in a study comparing EPA and DHA uptake from krill oil and fish oil in healthy male, though not conclusive, showed a tendency to increased krill oil incorporation into plasma phospholipids (62,63).

Krill hydrolysate

Krill is also an important source of high-quality marine protein with essential amino acids. Krill hydrolysates contain high levels of free amino acids, such alanine, proline, arginine, glutamine, leucine, glycine (64). Peptides derived from krill are known for their antioxidant activities (65). krill meal has therefore been used extensively in aquaculture, pet feeds and as bait in studies with fish such as salmonids (64,66). Krill meal is made from whole ground krill and is a combination of high-quality protein that contains all essential amino acids and lipids (krill oil) that are rich in long chain omega-3-PUFA (66). Krill protein hydrolysate or krill hydrolysate (KH) is partially hydrolyzed isolate derived from a whole Antarctic krill and has surfaced as a novel food (67). Krill hydrolysate is obtained by partially hydrolyzing Antarctic krill meal using food-grade protease.

Recently, it has been suggested that some of the beneficial effects of seafood on health could originate from the protein fraction, and studies in rodents have shown that a number of fish hydrolysates have lipid lowering and antioxidant effects (68).

Not only different sources of oil, but also different protein sources have been found to affect body weight gain and metabolic health differently. For example, feeding rodents with protein from either chicken, cod or milk was found to result in marked differences in body weight and fat gain and feed efficiency (body weight gain relative to total energy intake) with higher increases in these measures particularly for chicken protein (69). Since krill could be an

important source of protein, it is of interest to determine how this protein might affect body fat storage and components of metabolic syndrome. Also, protein in the form of hydrolysate may contain bioactive proteins/peptides with potential health benefits (54,66) and such effects of krill protein hydrolysate could further motivate increased harvest of krill and krill products for human consumption.

Furthermore, another protein source from Krill has shown to modulate some components of metabolic health. Rats fed with phospholipid-protein complex (PPC) from krill has shown to significantly reduce plasma TAG and cholesterols and resulted in a more beneficial fatty acids composition in rats which may suggest an anti-atherogenic potential (70).

KRILL STUDIES

Studies have shown a documented effect on improved metabolic health in rats fed with krill oil and reduced weight gain. Improved peripheral insulin sensitivity, one of the measurable components of metabolic health, was documented in rats fed with dietary supplementation with bioactive phospholipid concentrates of krill oil, buttermilk fat globule membranes (BMFC), and a combination of both in aged rats. Results from this study showed that a dietary supplementation with KOC with or without BMFC improves peripheral and central insulin resistance (71). Furthermore, another study documented a significant decrease in weight gain after four weeks of dietary supplementation with krill oil (KO) (72). This study also documented reduced serum lipids after 4-week consumption of KO. Specifically, consumption of KO reduced TAG, Total cholesterol (TC) and LDL-cholesterols (LDL-C), while no changes were observed in HDL-cholesterol (HDL-C) (72).

Study done on mice has also shown to have effect on weight gain and metabolic health. Mice fed with Antarctic krill oil (KO) had less body weight gain and accumulated less fat in tissue such as adipose and liver (73). In addition, metabolic health was improved in mice fed with KO. Glucose metabolism was improved and a glucose tolerance of about 22% was documented. Additionally, dyslipidemia caused by high fat diet was partially improved as well to a certain degree by KO feeding with a significant reduction of serum low density lipoprotein-cholesterol (LDL-C) content (73).

Krill powder, which contains both lipids and proteins has been documented to reduce plasma TAG level with concurrent increase in plasma bile acid (BA) level and thus improve metabolic health in high-fat fed mice. In this study, mice were fed with a high-fat (21% w/w) diet which consists of 20% crude protein (w/w) such as casein (control group) or krill protein hydrolysate (KPH) for 6 weeks (54). Results from this study show that dietary treatment with KPH reduced plasma TAG and non-esterified fatty acids (NEFAs). Since high plasma TAG concentration is a characteristic feature of the metabolic syndrome (MetS) (54), reduction in plasma TAG will thus reduce MetS phenotype. Furthermore, bile acid (BA) administration has been shown to reduce plasma TAG levels in animals and humans with hypertriglyceridemia (54). Thus, increase in BA in mice when fed with KPH should reduce components of metabolic syndrome and thus improve metabolic health. Another documented effect of KPH was decreased average body weight over the test period in mice fed KPH, whereas the mice fed casein gained weight

KRILL STUDIES ON HUMANS

Studies conducted on humans have shown improvement in some components of metabolic syndrome following supplementation with krill oil. Meta-analysis of randomized controlled trials (RCTs) reported significant reduction in plasma TAG concentrations following krill oil supplementation (74), a similar result found in rats (72). However, the observed reduction in plasma TAG levels was found only in subset of RCTs with krill oil supplementation lasting for 12 weeks or more and not in the subset of RCTs lasting less than 12 weeks (74). Furthermore, a substantial reduction in plasma levels of LDL-C was observed with krill oil supplementation (74), a similar result found in mice (73). However, the effect of krill oil on LDL-C levels did not reach statistical significance in subsets of RCTs with certain dosage. But there was a substantial reduction in plasma levels of LDL-C in subsets of RCTs with krill oil supplementation of 12 weeks or more., but not in subsets of RCTs with krill oil supplementation that lasted less than 12 weeks (74).

AIMS AND OBJECTIVES

Krill has been proposed to be a potentially disease-treating source of bioactive FAs and other nutrients (55). However, few studies have investigated how krill oil and krill hydrolysate affect components of the metabolic syndrome. Potentially, the combination of krill oil and krill hydrolysate might have synergistic bioactive effects. In this project we therefore aimed to determine effects of krill oil, krill hydrolysate and the combination of these on total body weight, body fat and organ weights, as well as other key components of metabolic syndrome including plasma glucose, TAG and lipoprotein.

In a study of male Wistar rats challenged with a Western High-fat diet to induce obesity, the specific objectives were to determine whether krill oil and krill hydrolysate, independently and/or in combination, affect

1) Body weight, body fat and organs weights
2) Plasma glucose, TAG and lipoproteins
3) Plasma and liver fatty acid composition

We hypothesized that both nutrient sources from krill would improve the measured components of the metabolic syndrome, with synergistic effects when combined.

MATERIALS AND METHODS

ANIMAL STUDY

The animal experiment began July 5th 2021 and was authorized by the Norwegian Food Safety Authority (Forsøksdyrforvaltningens tilsyn og søknadssystem (FOTS) permit number: 19553). 72 male Wister rats (strain is RjHan:WI) that were 5 weeks of age were purchased from Naiser AS in Norway but came originally from France. The rats were randomly distributed in 9 groups of 8 rats divided into two cages with four rats per cage. This thesis includes only 4 of the groups (**Figure 2.1**). Each group was therefore distributed into two cages (type IV cages, open). Then the cages were randomized to diet, whereby two cages per diet were given, i.e., 8 rats per diet. At start-up, one cage per diet was started on Monday and the rest of the cages started on Wednesday. When assigning the rats to the cages, block randomization (Research Randomizer) was used to determine placement in the cages and their order at the day of euthanasia. The rats were labeled with a marker on the tail to distinguish them.

The rats were acclimatized to their surroundings from their arrival (June 18th 2021) to the start of the study (July 5th 2021). They were kept in a 12-hour day/night cycle at a constant temperature of 22 ± 2 °C and a humidity of 55 ± 5 % in line with the Guidelines for the Care and Use of Experimental Animals, and the practice of Norwegian Law and regulations on experiments with live animals. They were provided free access to water during the whole experiment as well as sufficient but controlled access to the diet. The animal cages were changed and cleaned once a week.

The study design included in this thesis involves controlled feeding experiments in the rats, to determine effects of krill oil (K), krill hydrolysate (KH), and the combination of krill and krill hydrolysate “Pristine” on a high-fat (HF) “Western” diet background, compared to a control group, consuming only high-fat diet but the same total energy, oil and protein (**Table 2.2)**. In the experimental groups, krill oil replaced soybean oil and krill hydrolysate replaced casein, while the contents of carbohydrates, fiber and other nutrients were designed to constant across all groups. Additionally low-fat (LF) (product code: T-5755-6991, testdiet.com) control diet was administered to another group to control the effect of the HF diet.

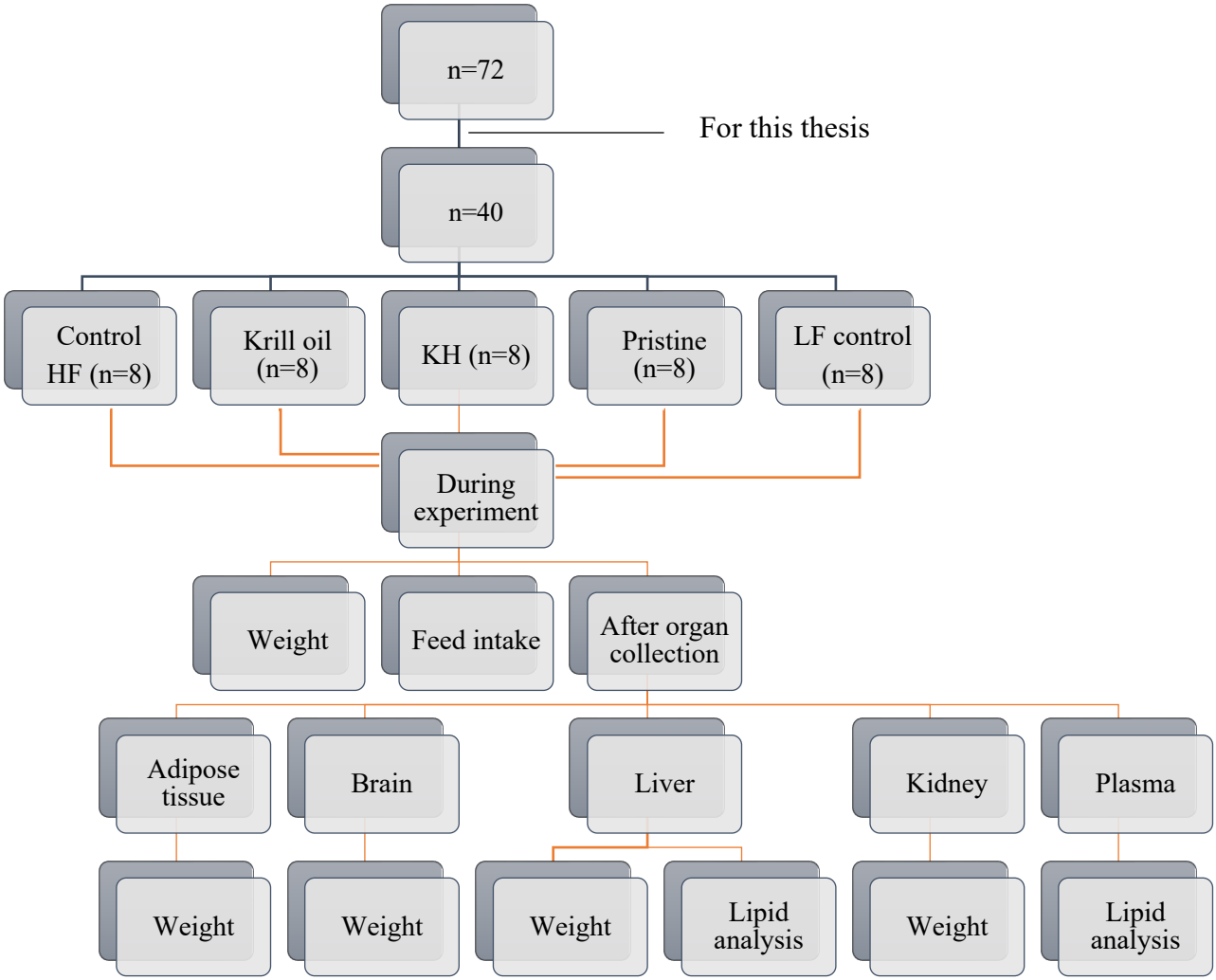

Figure 2.1: Illustration of the experimental design of the study.

Table 2.1: Cage distribution and type of diet provided for this thesis

Group	**Cage number**	**Number of animals**	**Dietary intervention**
1	1 2	4 4	HF – control HF – control
2	3 4	4 4	HF+ K HF+ K
3	5 6	4 4	HF + KH HF + KH
4	7 8	4 4	HF+PRI HF+PRI

5		4 4	LF- control LF- control

Abbreviations: HF, high fat; K, krill oil; KH, krill hydrolysate; PRI, pristine; LF, low fat.

DIET PREPARATION AND DAILY PROCEDURE

The feed was prepared by mixing all the dry ingredients in a bowl, first by hand, then in a mixer/food processor, at the lowest speed (indicated as 1) for approximately 2-3 minutes and then evenly increasing the speed to highest (indicated as 4) for approximately 3-4 minutes. Oil (amount according to **Table 2.2**) was then gradually added while the blender was at the lowest speed, then the speed was evenly increased till the oil was properly mixed with the dry feed. Water (amount according to **Table 2.2)** was then added inn and mixed same way as was done for oil. The finished-mixed diet was transferred to another bowl, weighed, and then stored in prelabeled plastic bags (content, quantity, and date) and was refrigerated until used. The total feed composition is presented in **Table 2.2**. The feed preparation was done by Thomas Aquinas Aloysius from the Lipid Research group at the University of Bergen.

During the 8-weeks intervention, the rats were fed 333 g of feed three times a week except for the first week of the experiment where they were fed only twice a week. In week 0 and 4 of the experiment the leftover feed in the cages was weighed and feed consumed per cage was calculated. Weighing of the animals was done at the start of the experiment (week 0), then once a week during the experiment and on the day of sacrifice.

Table 2.2: Feed composition per 1000 g feed.

	Western diet (HF)		**1-high fat control**	**2-krill oil (KO)**	**3-krill hydrolysat-pulver (KH)**	**4-pristine (KO+KH)**
	Kcal	**Energy%**	**g in diet**	**g in diet**	**g in diet**	**g in diet**
Protein	1182.3	24.2%	295.6	295.6	295.6	295.6
Fat	2362.9	48.3%	262.5	262.5	262.5	262.5
Carbohydrate	1344.4	27.5%	388.3	388.3	388.3	388.3
Micronutrients	0.0	0.0%	53,6	53.6	53.6	53.6
Weights in feed (g/kg)						
Casein in diet			239.1	275.9	275.9	275.9
Added Casein[1]			99.6	99.9		

Added Soy oil			43.8		43.9	
Pristine						143.8
Krill oil				45.2		
Krill hydrolysate					101.1	
Soy oil			20.8	20.8	20.8	20.8
Lard			195.0	194.8	194.8	195.1
Carbohydrates						
Cornstarch			93.9	93.9	93.9	93.9
Maltodextrin/dextrose			137.9	137.9	137.9	137.9
Sucrose			104.3	104.3	104,3	104.3
Fiber			52.2	52.2	52.2	52.2
Micronutrients		HF				
AIN-93G-MX mineral mix		42.8	37.21	37.21	37.21	37.21
AIN-93-VX vitamin mix		12.2	10.61	10.61	10.61	10.61
L-Cystine		3.8	3.19	3.19	3.19	3.19
Choline bitartrate		3.0	2.61	2.61	2.61	2.61
tert-Butyl-hydroquinone		0.017	0.015	0.015	0.015	0.015
Vann i proteinkilde			**33.9**	**33.9**	**23.9**	**23.9**
Total			1040.3	1078.4	1078.3	1077.4
Dry weight		100.0%	**1000.0**	**1000.0**	**1000.0**	**1000.0**

Casein[1] (Arne B Corneliussen, artikkel no: 4815007480), Western diet (product code: T-5TJN-1810850, testdiet.com), Krill oil (Batch no: 208920, Rimfrost, Ålesund, Norway), Krill hydrolysate (Batch no: 231120, Rimfrost, Ålesund, Norway).

EUTHANASIA AND TISSUE COLLECTION

At the end of study, the rats were euthanized under fasting conditions in the order determined by the block randomization at the start of the study. The euthanasia phase lasted for 4 days, in which 72 rats, two from each of the cage, were euthanized per day. The rats were anesthetized with 5% sevoflurane (Zoetis Belgium SA, Belgium) and then transferred to a mask with 2-3% sevoflurane. They were then sacrificed by drawing blood from the heart using 3 ml EDTA vacutainers and standard blood sampling equipment. The EDTA tubes containing blood were placed on ice, before being centrifuged at 2000 G for 15 minutes at 4 °C. EDTA plasma was then isolated, aliquoted and stored in a freezer at -80 °C. Organs, heart, liver, epididymal fat, perirenal fat, mesenteric fat, kidney, brain, interscapular brown fat and inguinal white fat were

removed and weighed and immediately freeze-clamped in liquid nitrogen and placed directly on dry ice before being stored in a freezer at -80 °C until needed for further analysis.

ANALYSIS OF GLUCOSE AND PLASMA LIPIDS

Analysis of plasma lipids was carried out by technicians in the hospital laboratory of Helse Bergen at the department of Medical Biochemistry and Pharmacology (MBF). The analyzer machine used was Cobas 8000 c702 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Absorbance was measured photometrically endpoint analysis to detect the colored Trinder reaction product. The intensity of the color is directly proportional to the lipid/glucose concentration in the sample which is determined by measuring the increase in absorbance. The concentration of plasma lipids was then calculated as mmol/liter plasma on an internal standard curve.

Table 2.4: Reagent kits for plasma lipid and glucose analysis

Reagents	Catalog no.	Manufacturer	Wavelength
TAG	0517407190	Roche Diagnostics AS	505 nm
Cholesterol	05168538190	Roche Diagnostics AS	505 nm
HDL-C	07528582190	Roche Diagnostics AS	600 nm
LDL-C	07005768190	Roche Diagnostics AS	600 nm
Glucose	0516879190	Roche Diagnostics AS	340 nm

Reagents supplier was Roche Diagnostics AS (GmbH, Mannheim, Germany). Abbreviations: TAG, triacylglycerol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

QUANTIFICATION OF PLASMA AND LIVER FATTY ACIDS

Measurement of FA composition in plasma and liver extract using GC-LC was carried out by Pavol Bohov from the Lipid Research group at the University of Bergen. Sample preparations leading up to the GC-analysis that are described below were carried out by Kari Helland Mortensen from the Lipid Research group at the University of Bergen.

Lipid extraction

This procedure was performed by laboratory technician, Kari Helland Mortensen from the Lipid Research Group at the University of Bergen. Approximately 150 mg frozen liver tissue was

added 5 ml chloroform-methanol and homogenized using Ultra-Turrax T25 homogenizer (IKA® - Werke, GmbH & Co. KG, Staufen, Germany). The lipids were extracted with chloroform-methanol and a volume equivalent to 3 mg of the extract was sent for analysis of FA composition.

Procedure for preparation of derivatization, internal standard, and neutralization solutions.

Schott bottles washed with RBS, distilled water, HPLC water, methanol, chloroform, hexane, and dried nitrogen were used to prepare the solutions. For the derivatization solution, 50 ml Schott bottle was added methanol HPLC (49 ml) and sulfuric acid 95-97% (1 ml), then shaken for 10 seconds. Furthermore, for the internal standard solution, 100 ml Schott bottle was added toluene HPLC (98.75 ml) and 1.25 ml solution of 1 mg C21:0/ml toluene HPLC and shaken for 10 seconds. Lastly, for the neutralization solution in a 100 ml Schott bottle was added 1.5 M K_2CO_3 (20.732 g) and HPLC water (95.0 ml), then the mixture was shaken until dissolution. The prepared solutions were then used after temperature equilibration to 20 °C.

Lipid Pre-esterification with H_2SO_4-methanol

For the pre-esterification of plasma lipids, a 2 ml glass vial (Chromacol 2-SV) with a cap (8-SCS) and a seal (8-ST14) was added 20 µl plasma, derivatization solution (0.4 ml) and internal standard solution (0.4 ml), then vortexed for 5 seconds. The samples are then heated at 90 °C for 65 minutes to obtain FA- methyl esters (FAME). The samples were then cooled off and 0.3 ml HPLC and 120 µl neutralization solution was added. The vials were closed and centrifuged (3100 rpm, 5 minutes, 20°C). Next, about 200 µl of the upper toluene layer was transferred into another vial (ALS 03-FISV) with caps (Chromacol 9-SC(b)-ST1) and was tightly closed, level marked, and the content mixed by turning 3 times and vortexed for 5 seconds. For the pre-esterification of liver lipid extract, a volume equivalent to 3 mg liver extract was evaporated with nitrogen gas and the 20 µl plasma was replaced by 20 µl water, thereafter, the exact same steps for pre-esterification of plasma described above was followed.

Gas liquid chromatography (GC) analysis

FAME was then analyzed by GC using gas chromatograph (GC 8000 TOP, Finnigan, USA) that has an inbuilt programmed temperature evaporation injector, flame ionization detector, AS 800 autosampler and a fused silica capillary column DBI-ms (J & W Scientific, USA). A carrier

gas of hydrogen was used. The temperature of the column was set (110 to 310°C) with 2.5°C/min gradient. GC signal was captured and assessed using Chromeleon software (Dionex Corporation, USA). The peaks were identified using known FA standards and mass spectra attained by GC/MS analysis (GCQ, Finnigan, USA) that was done on the same column. Internal standard was used for quantifying after calibration with known mixtures of FA-standards. Values of FA in plasma and liver were presented as (wt%) (75).

STATISTICAL ANALYSIS

Collected raw data that were relevant for this thesis was extracted and organized in Microsoft Excel for Mac version 16.59. The organized data were then transferred to Graph-Pad prism software version 9 where statistical analysis was performed. The results were presented as means with standard deviation. One-way ANOVA with Dunnett's and Tukey's multiple comparison test was used to analyze the difference between the intervention groups compared to control (high fat (HF)) or between the groups respectively. When comparing the effect of HF to LF, unpaired two-tailed *t*-test was used to determine statistical difference between the means. The level of statistical significance was defined as $p < 0.05$. Graph-Pad prism was also used to produce the illustrated graphs. Microsoft Excel for Mac version 16.59 was used for the analysis of plasma and liver FAs shown in **Table 3.1-3.3**.

RESULTS

Rat body weight gain

After 8 weeks of dietary intervention, body weight gain was observed in all groups, with a greater growth in the animals receiving the combination of K and KH diet in comparison to those who were receiving the individual diets (**Figure 3.1 A**). Significant difference (P=0.0155) was detected between the HF group (control) and the combination group of K and KH (K+KH), as well (P=0.0240) between the K and K+KH groups (**Figure 3.1 B**). For body weight gain (weight measured at the end of study – weight measured at the start of study), a significance difference was detected (P=0.0288) between HF and the K+KH group with rats fed K+KH showing higher body weight gain (**Figure 3.1 C**).

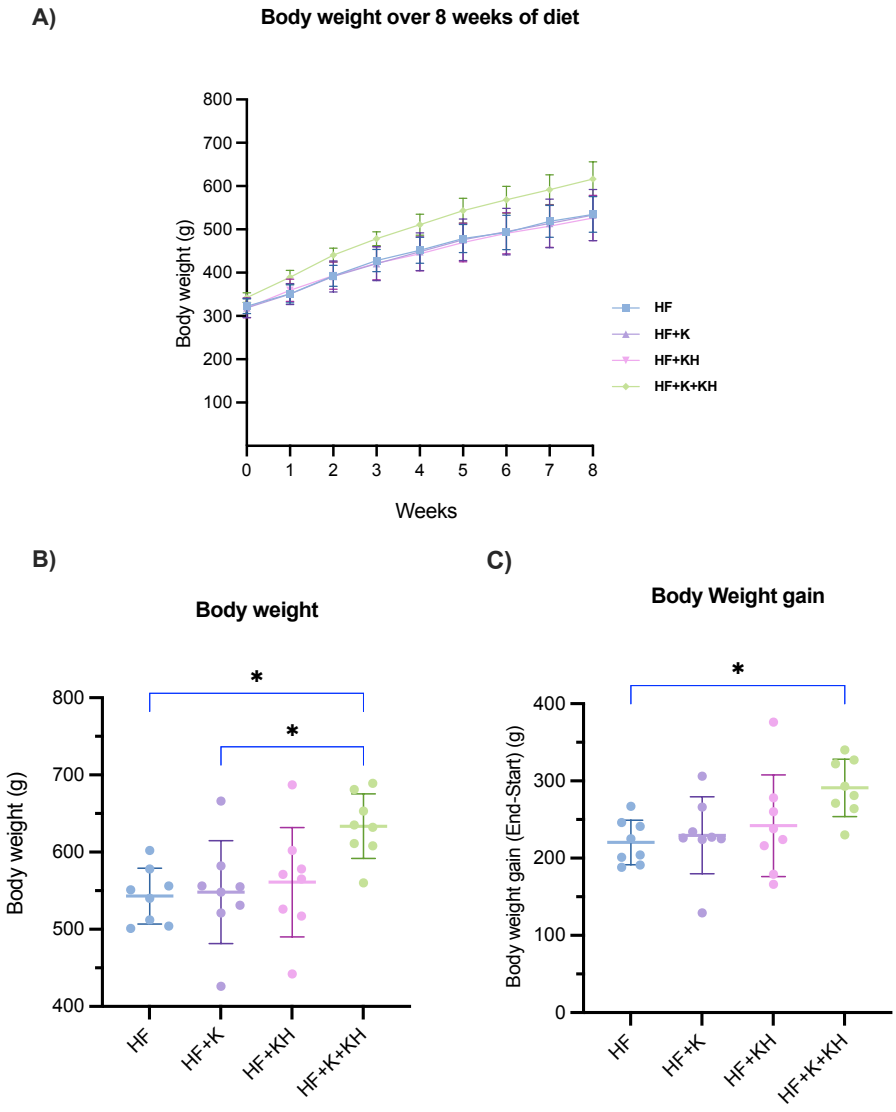

Figure 3.1: A: Body weight of the rats (n=8) measured in grams over a period of 8 weeks. **B**: Rat body weight (g) measured at the end of study (day of sacrifice). **C:** Body weight gain calculated as the difference in body weight at the start and the end of the study. The results are presented as means with standard deviation (n=8). GraphPad prism was used to analyze the data. The statistical method used is one-way ANOVA with Tukey's multiple comparisons test (**B** and **C)**. Statistically significant differences between the groups are indicated with asterisk: *p<0.05. Abbreviation: HF, high fat diet; K, Krill oil; KH, Krill hydrolysate.

HF control vs LF control on body weight

When comparing the body weight effect of HF diet to LF diet over an 8-week period of dietary intervention, slight increase was seen on the rats that received HF diet (**Figure 3.2 A**). This was also reflected in the body weight gain of the rats in (**Figure 3.2** C) where no significance difference was detected among the groups, but with a slightly higher mean weight value on HF-group (220g) than the LF-group (203g). Similarly, no significant difference in the body weight (g) was detected between the diet groups (**Figure 3.2 B**). However, when measuring the organs weight (g), a significant difference in liver weight was observed between the control groups (P=0.005) (**Figure 3.2 D**).

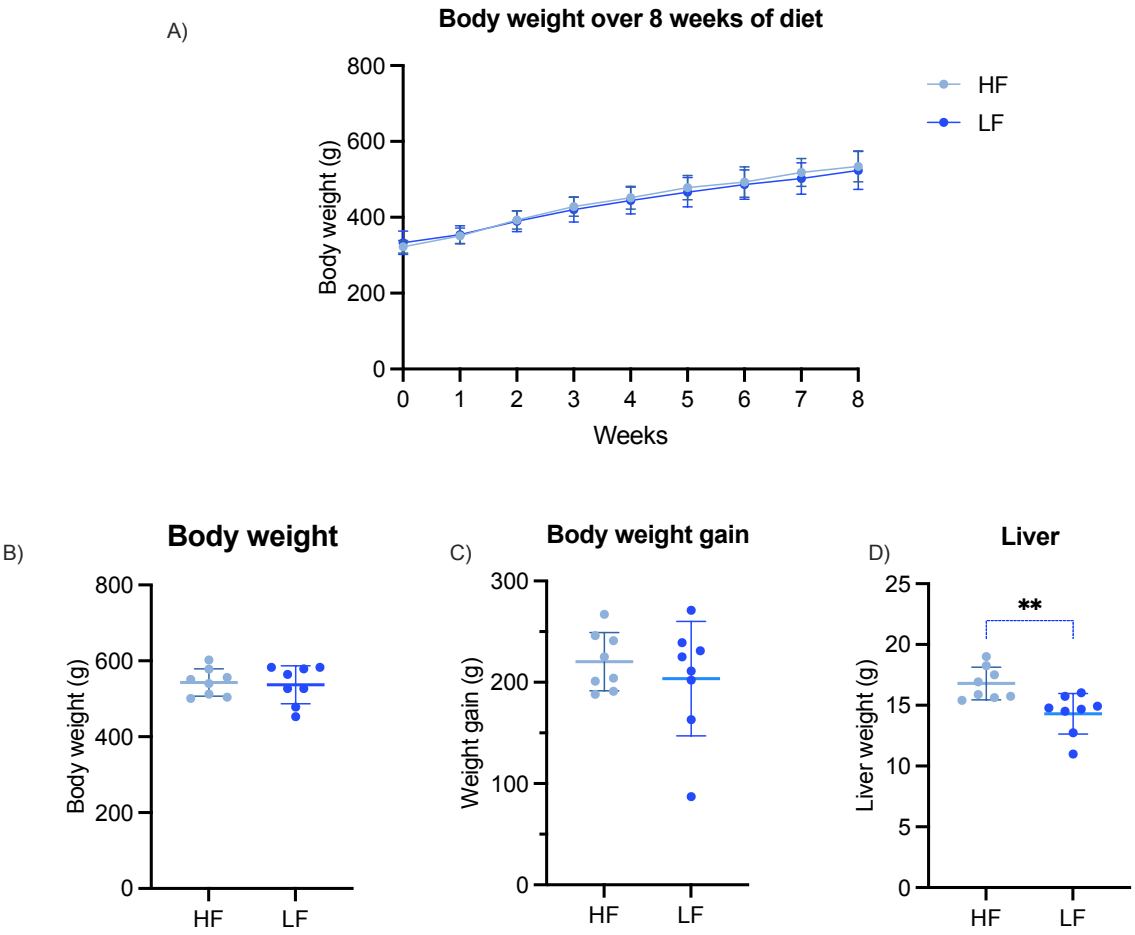

Figure 3.2 A-D: Body weight of the rats (n=8) measured in grams over a period of 8 weeks. **B**: Rat body weight (g) measured at the end of study (day of sacrifice). **C:** Body weight gain calculated as the difference in body weight at the start and the end of the study. **D:** Liver weight measured at the day of sacrifice. The results are presented as means with standard deviation (n=8). GraphPad prism was used to analyze the data. Unpaired two-tailed *t*-test was used to evaluate statistical differences between the control diet groups. Statistically significant group difference is denoted with asterisk **P<0.01. Abbreviation: HF, high fat diet; LF, low fat diet.

Organ weights

When comparing liver weights between the animal group that received the intervention diet to those that received the control diet (HF), a significance difference (P=0.0055) was detected between HF and the combination of K+KH. The greatest significance difference was seen between the KH and K+KH groups (P=0.0002). Furthermore, a significant difference was also observed between K and K+KH (P=0.0376) (**Figure 3.3 A**). When it come to the liver weight/body weight ratio, no significance is difference is detected between the HF-control and the rest of the diet groups (**Figure 3.3 B)**. No significant weight difference was detected between the groups in both heart and kidney (**Figure 3.3 C and B)**.

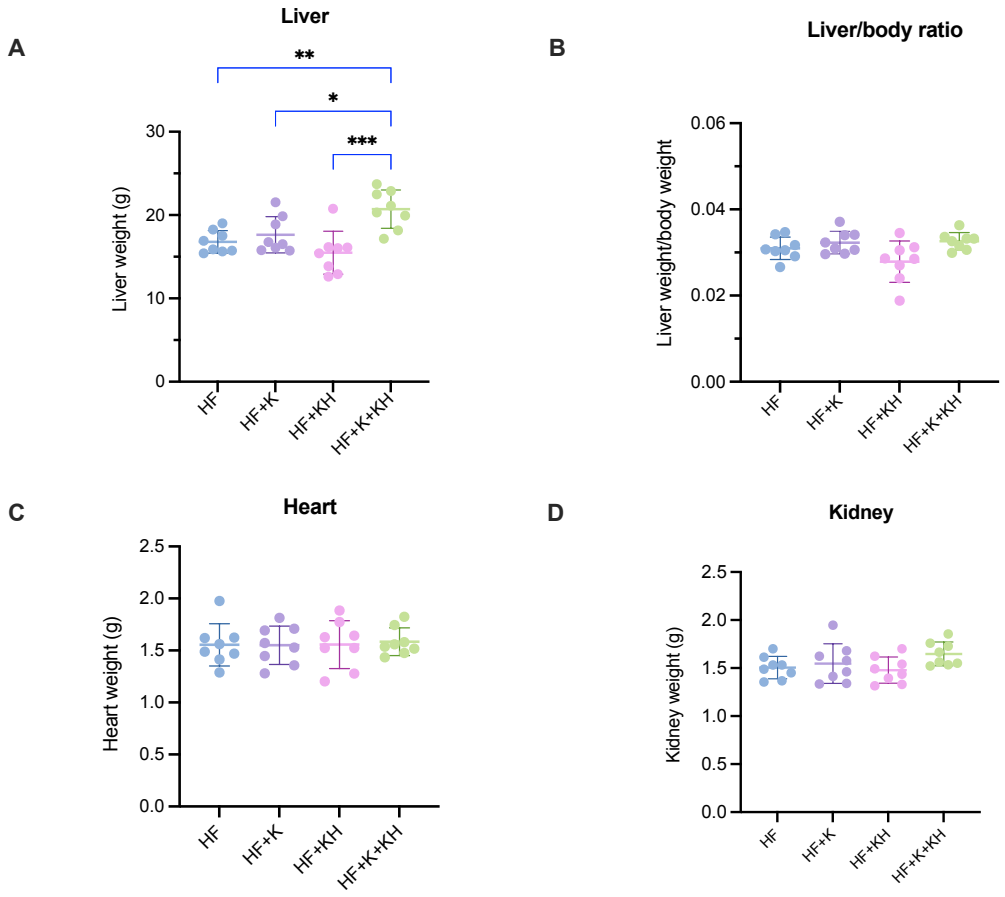

Figure 3.3 A-D: A, C & D: Organ weight of the rats (n=8) measured in grams on the day of sacrifice. **B**: Liver and body weight ratio calculated as liver weight/ body weight. The data was analyzed in GraphPad prism. The values are presented as mean with standard deviation. Statistical methods used are one-way ANOVA with Tukey's multiple comparisons test (**A, C & D**) and Dunnett's multiple comparison test (**B**). Statistical significance difference compared to respective means and between the groups, are demonstrated with asterisks: * P<0.05, ** P<0.01 and *** P<0.001. Abbreviation: HF, high fat diet; K, Krill oil; KH, Krill hydrolysate.

Adipose tissue weight

In the case of adipose tissues, significance weight differences were observed only in WAT tissues (**Figure 3.3**). The combination diet K+KH showed significantly higher weights of mWAT, pWAT and eWAT, and iWAT compared to the control group (P=0.010, P=0.041, P=0.020 and P=0.042, respectively) (**Figure 3.3A-D**). Significant differences were also detected between K and K+KH (P=0.011 in mWAT, P=0.005 in pWAT and P=0.012 in eWAT), and between the KH and K+KH groups (P=0.012 in mWAT, P=0.0001 in pWAT and P=0.035 in eWAT (**Figure 3.3 A-D**). No significant weight differences between the groups were observed in iBAT (**Figure 3.3E**).

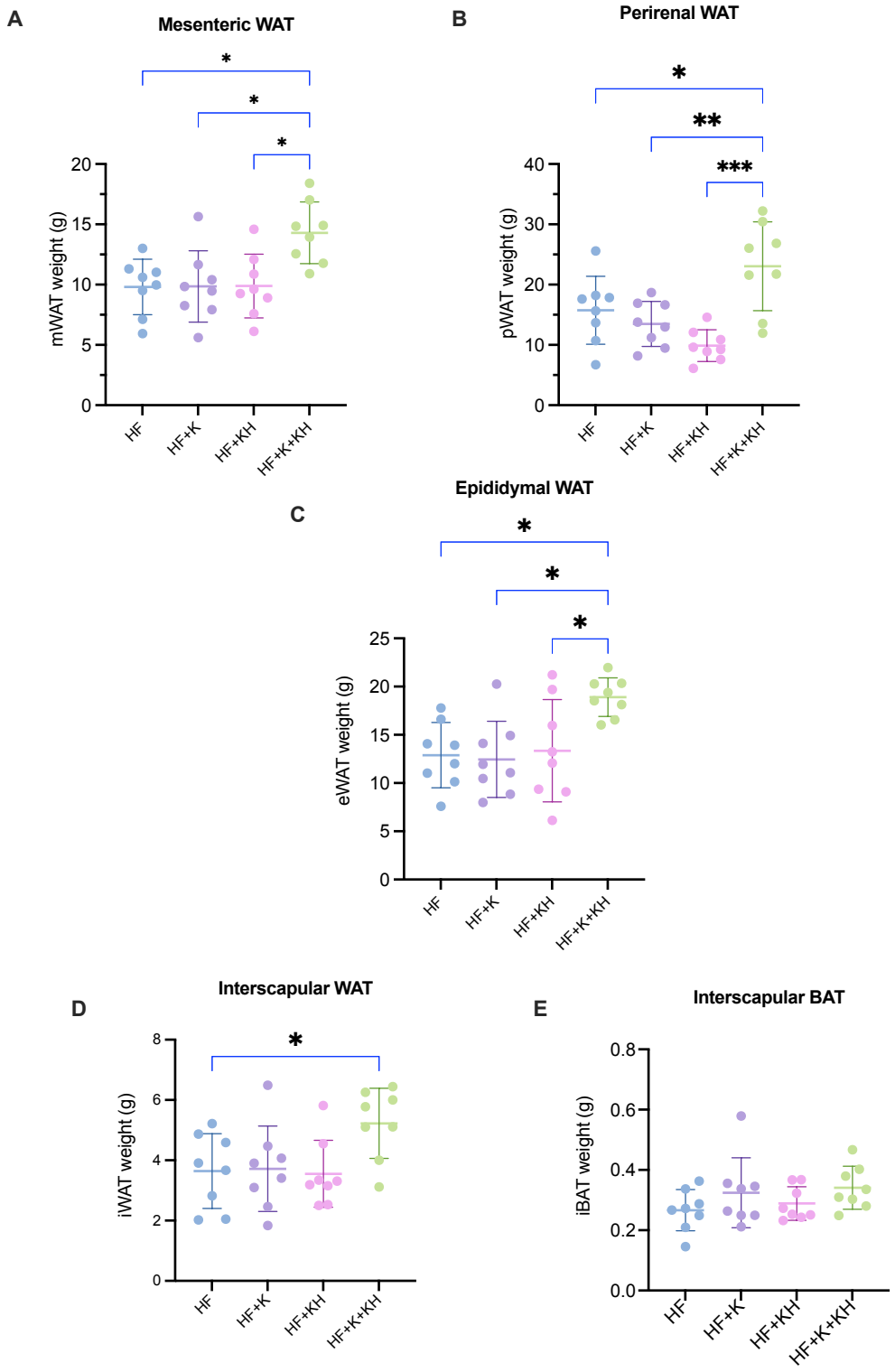

Figure 3.4 A-E: Weight of adipose tissue measured in grams (g) at the day of sacrifice (n=8). The results are presented as mean with standard deviation. The data were analyzed in GraphPad prism. Statistical methods used are one-way ANOVA with Tukey's multiple comparisons test (**A-C**) and Dunnett's multiple comparison test (**D-E**). Statistical significance difference

compared to respective means and between the groups, are demonstrated with asterisks: * P<0.05, ** P<0.01 and *** P<0.001. Abbreviation: HF, high fat diet; K, Krill oil; KH, Krill hydrolysate.

HF control vs LF control on fat tissue weight

Comparison of HF and LF diet on the different adipose tissues (**Figure 3.5**) were statistically insignificant.

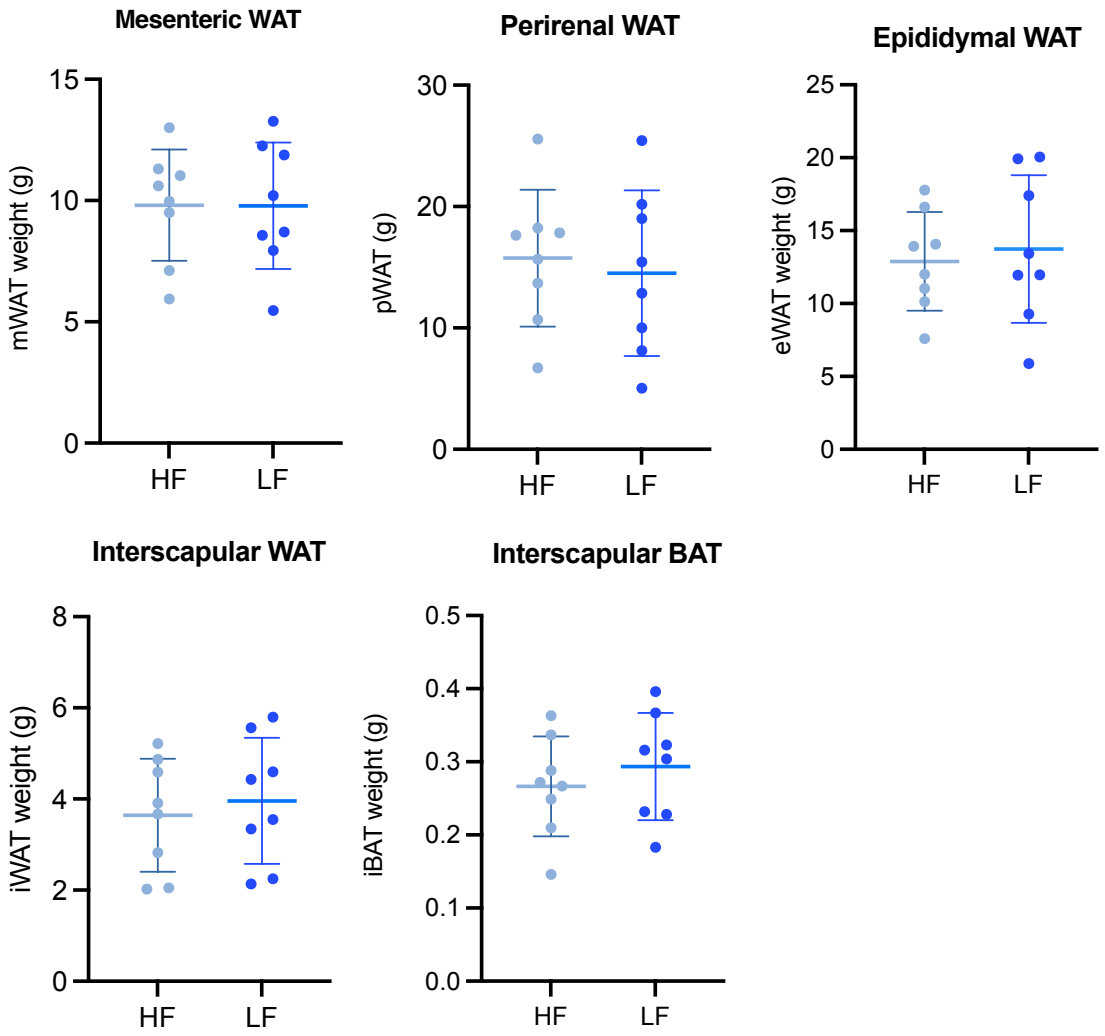

Figure 3.5: Fat tissue weights (g) of the rats (n=8) receiving control HF and LF diet.
The results are presented as means with standard deviation (n=8). GraphPad prism was used to analyze the data. Unpaired two-tailed *t*-test was used to evaluate statistical differences between the control diet groups. Abbreviation: HF, high fat diet; LF, low fat diet

Plasma lipids

Interestingly for the concentration of plasma lipids groups, there were no significant differences detected among the dietary groups after 8 weeks of the intervention. The plasma concentration of TAG was lowest in the rats that received KO diet compared to the other diets. Meanwhile, the concentration of plasma HDL seems to be almost similar in all the diet groups. Moreover, plasma LDL concentration is highest in the rats that received pristine diet (KO+KH), while it remained almost the same for the rest of the diet. For plasma cholesterol concentration, rats that received KO diets seem to have slightly lower cholesterol than the rest of the diet groups while for the rats that received pristine seem to have highest plasma cholesterol concentration (**Figure 3.5)**.

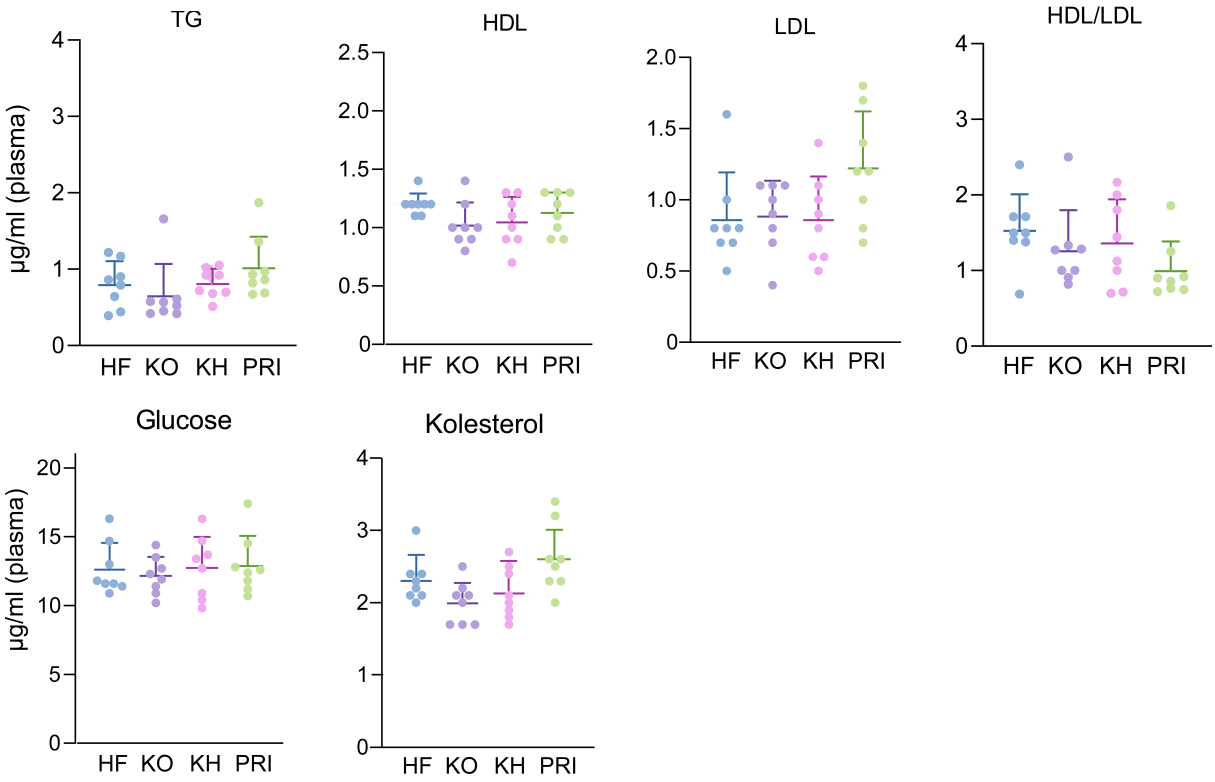

Figure 3.6: Plasma lipid concentration of male Wistar rats (n=8) after 8 weeks of dietary intervention. TG; triacylglycerol; HDL, high density lipoprotein; LDL, low density lipoprotein; HF, high fat; KO, krill oil; KH, krill hydrolysate; PRI, pristine.

Fatty acid composition in plasma and liver

As expected, the proportion of total omega-3 PUFA (wt%) in both plasma and liver after 8 weeks of dietary intervention were very high in the rats that were fed KO and KO+KH as "Pristine" and lowest in those who had received KH diet. The omega-3 PUFA values observed in the liver were slightly higher than those that were in plasma in all the diet groups (**Table 3.1 and 3.2)**. When comparing the diet groups to HF (control) of plasma omega-3 PUFA, significance difference was detected between KO and HF ($P<0.0001$), and pristine and HF ($P<0.0001$) (**Figure 3.5 A)**. Same significant difference between the groups of (HF vs. KO and HF vs. PRI) was observed for hepatic omega-3 PUFA concentrations (**Figure 3.5 B**).

Furthermore, the total omega-6 PUFA (wt%) in plasma was highest in HF and KH diet (**Table 3.1**). In liver on the other hand, omega-6 PUFA values in all the diet groups were slightly lower than those in plasma (**Table 3.2**). For total omega-6 concentrations in plasma, significant difference in plasma omega-6 PUFA was identified between HF and KO group ($P<0.0001$) and between HF and PRI group ($P=0.046$) (**Figure 3.5 E**) while in liver significant difference was only observed between HF and KO ($P=0.003$) (**Figure 3.5 F**).

Total SFA proportions in plasma (wt%) were almost similar in all the groups (**Table 3.1)** and therefore, for the concentration of SFAs (μg/ml plasma), no significant difference was detected among the groups (**Figure 3.5 G)**. Same can be said about total FA concentrations whereby only slightly higher concentrations were seen on the pristine group but no significance difference observed among the groups (**Figure 3.5 I)**. On the other hand, in comparison to the HF diet, pristine resulted in a significantly higher concentration of hepatic SFA ($P=0.002$) (**Figure 3.5 H**) and Total FA ($P=0.015$) (**Figure 3.5 J**).

As for the specific omega-3 PUFAs, EPA, DHA and DPA in plasma and liver, significant differences were detected in KO and pristine groups which correlated with the proportion of total omega-3 PUFA (**Table 3.3**). Fittingly, the concentrations of plasma and liver EPA, DHA, and DPA were lowest in the rats that had received HF and KH diet (**Table 3.1 & 3.2**). Compared with HF, KO and pristine diets resulted in significantly higher concentrations of EPA (C20:5n-3), DHA (C22:6n-3), DPA (C22:5n-3). HF and KH as shown in **Table 3.3** and **Figure 3.6 A-D**.

Table 3.1: Fatty acid composition (g/100 g total fatty acids) in plasma of male Wistar rats after 8 weeks of dietary intervention.

	Plasma (wt%)							
Fatty acids	**HF (n=8)**	**+/- SD**	**KO (n=8)**	**+/- SD**	**KH (n=8)**	**+/- SD**	**PRI (n=8)**	**+/- SD**
Total FA	100	0	100	0	100	0	100	0
SFA	30.4	1.01	31.7	0.609	31.1	1.17	31.0	1.04
MUFA	16.8	2.97	17.6	1.63	17.5	2.67	21.1	1.73
PUFA n-3	**5.38**	**0.338**	**16.0**	**0.797**	**4.88**	**0.660**	**11.6**	**1.09**
PUFA n-6	46.9	2.32	34.2	2.35	46.1	1.90	35.9	1.25
PUFA n-9	0.155	0.015	0.110	0.015	0.140	0.015	0.120	0.013
Trans-FA	0.324	0.022	0.310	0.030	0.350	0.025	0.330	0.022
PUFA n-3/ PUFA n-6	0.115	0.010	**0.470**	0.051	0.110	0.014	**0.320**	0.028
C10:0	0.019	0.010	0.027	0.018	0.020	0.009	0.024	0.012
C12:0	0.057	0.023	0.089	0.041	0.071	0.024	0.075	0.036
C14:1n-5	0.027	0.005	0.040	0.012	0.032	0.009	0.037	0.010
C14:0	0.513	0.101	0.722	0.242	0.590	0.141	0.766	0.215
C15:0	0.220	0.022	0.258	0.024	0.232	0.029	0.247	0.028
C16:1n-9	0.157	0.039	0.155	0.031	0.142	0.029	0.189	0.028
C16:1n-7	1.14	0.422	1.36	0.224	1.16	0.453	2.04	0.430
C16:1n-7t	0.018	0.003	0.021	0.004	0.018	0.003	0.023	0.003
C16:1	0.033	0.007	0.058	0.011	0.039	0.012	0.059	0.009
C16:0	15.8	0.581	17.7	0.888	15.8	0.504	17.5	1.130
C17:0	0.259	0.016	0.257	0.009	0.264	0.018	0.250	0.011
C18:3n-6	0.235	0.033	0.134	0.014	0.225	0.061	0.155	0.024
C18:4n-3	0.026	0.009	0.125	0.064	0.033	0.016	0.067	0.022
C18:2n-6	20.2	2.08	21.4	1.175	22.3	3.38	19.5	1.45
C18:3n-3	0.916	0.132	1.010	0.186	1.07	0.196	0.961	0.133
C18:1n-9	12.3	2.24	12.3	1.293	12.7	1.90	14.9	1.28
C18:1n-7	2.14	0.359	2.450	0.194	2.28	0.464	2.69	0.259
C18:1t	0.310	0.021	0.292	0.027	0.329	0.023	0.307	0.020
C18:1	0.290	0.017	0.322	0.024	0.327	0.018	0.333	0.015
C18:0	12.30	1.20	11.4	0.782	12.8	1.50	10.9	0.733
C20:4n-6	24.70	3.73	11.3	2.595	21.9	4.21	14.9	1.94
C20:5n-3	**0.488**	**0.124**	**7.10**	**1.331**	**0.522**	**0.078**	**3.68**	**0.609**
C20:3n-9	0.155	0.015	0.113	0.015	0.145	0.015	0.116	0.013
C20:3n-6	0.698	0.144	1.010	0.188	0.760	0.177	0.947	0.114
C20:4n-3	0.115	0.009	0.188	0.025	0.110	0.011	0.142	0.011
C20:2n-6	0.327	0.059	0.223	0.023	0.338	0.066	0.250	0.018
C20:1n-11	0.037	0.007	0.035	0.010	0.034	0.010	0.034	0.005
C20:1n-9	0.156	0.041	0.134	0.039	0.152	0.034	0.170	0.025

C20:1n-7	0.221	0.040	0.273	0.079	0.365	0.110	0.217	0.041
C20:0	0.090	0.012	0.084	0.016	0.101	0.019	0.086	0.008
C21:5n-3	**0.000**	**0.000**	**0.096**	**0.025**	**0.000**	**0.000**	**0.055**	**0.012**
C22:5n-6	0.198	0.044	0.040	0.005	0.160	0.070	0.040	0.006
C22:6n-3	**3.18**	**0.312**	**5.70**	**0.796**	**2.58**	**0.832**	**5.22**	**0.478**
C22:4n-6	0.435	0.061	0.090	0.015	0.360	0.079	0.140	0.014
C22:5n-3	0.660	0.103	1.82	0.186	0.570	0.105	1.47	0.300
C22:2n-6	0.015	0.003	0.020	0.003	0.020	0.005	0.014	0.004
C22:1n-11	0.002	0.001	0.002	0.002	0.002	0.001	0.003	0.001
C22:1n-9	0.010	0.001	0.050	0.018	0.013	0.002	0.040	0.011
C22:1n-7	0.035	0.006	0.070	0.016	0.060	0.022	0.050	0.010
C22:0	0.194	0.040	0.180	0.032	0.190	0.034	0.150	0.017
C23:0	0.138	0.027	0.130	0.026	0.120	0.018	0.100	0.017
C24:1n-9	0.215	0.045	0.350	0.079	0.190	0.023	0.280	0.058
C24:0	0.410	0.079	0.440	0.095	0.410	0.063	0.360	0.060

Values are mean ±SD. HF, high fat; KO, krill oil; KH, krill hydrolysate; PRI, pristine (KO+KH); SFA, saturated fatty acids; FA, fatty acids; MUFA, monounsaturated fatty acids; PUFA n-3/-6, omega-3/-6 polyunsaturated fatty acids.

Table 3.2: Fatty acid composition (g fatty acids/100 g total fatty acids) in liver of male Wistar rats after 8 weeks of dietary intervention.

	Liver (wt%)							
Fatty acids	**HF (n=8)**	**+/-SD**	**KO (n=8)**	**+/-SD**	**KH (n=8)**	**+/-SD**	**PRI (n=8)**	***+/-SD**
Total FA	100	0.000	100	0.000	100	0.000	100	0.000
SFA	26.6	0.536	26.8	1.75	28.1	2.03	27.0	1.46
MUFA	28.5	3.70	24.7	3.38	27.8	3.56	31.7	3.14
PUFA n-3	**6.16**	**0.997**	**21.0**	**1.36**	**5.70**	**0.531**	**13.7**	**3.22**
PUFA n-6	38.3	2.75	27.1	1.68	37.9	2.72	27.3	1.13
PUFA n-9	0.124	0.029	0.085	0.013	0.120	0.021	0.084	0.007
Trans-FA	0.325	0.014	0.291	0.020	0.320	0.026	0.277	0.018
PUFA n-3/ PUFA n-6	**0.160**	0.016	**0.778**	0.064	**0.151**	0.011	**0.499**	0.105
C10:0	0.004	0.001	0.003	0.001	0.004	0.001	0.003	0.001
C12:0	0.022	0.004	0.015	0.003	0.024	0.005	0.019	0.002
C14:1n-5	0.028	0.008	0.021	0.004	0.034	0.017	0.032	0.008
C14:0	0.648	0.091	0.586	0.115	0.709	0.173	0.786	0.135
C15:0	0.238	0.018	0.243	0.023	0.251	0.028	0.275	0.026
C16:1n-9	0.323	0.064	0.279	0.060	0.295	0.055	0.370	0.075
C16:1n-7	1.72	0.635	1.54	0.415	1.79	0.795	2.72	0.691

C16:1n-7t	0.030	0.004	0.028	0.003	0.027	0.005	0.032	0.005
C16:1	0.063	0.012	0.073	0.010	0.070	0.024	0.088	0.015
C16:0	17.1	1.00	17.1	0.665	17.5	1.29	18.8	1.13
C17:0	0.212	0.014	0.222	0.017	0.216	0.026	0.200	0.018
C18:3n-6	0.379	0.063	0.139	0.021	0.303	0.048	0.195	0.026
C18:4n-3	0.056	0.013	0.098	0.024	0.049	0.012	0.069	0.013
C18:2n-6	24.5	1.25	20.0	0.971	24.0	2.55	19.8	1.52
C18:3n-3	1.31	0.163	1.46	0.243	1.37	0.290	1.40	0.262
C18:1n-9	22.6	2.78	19.4	2.70	21.6	2.33	24.2	2.00
C18:1n-7	2.88	0.492	2.49	0.345	3.038	0.688	3.37	0.583
C18:1t	0.294	0.018	0.263	0.021	0.293	0.028	0.244	0.022
C18:1	0.264	0.015	0.286	0.021	0.289	0.020	0.265	0.024
C18:0	7.58	1.00	7.68	1.87	8.495	1.98	6.04	0.934
C20:4n-6	11.3	1.83	5.61	1.57	11.57	2.31	5.97	1.17
C20:5n-3	**0.382**	**0.084**	**4.76**	**0.999**	**0.372**	**0.085**	**2.44**	**0.699**
C20:3n-9	0.124	0.029	0.085	0.013	0.120	0.021	0.084	0.007
C20:3n-6	0.741	0.226	0.896	0.220	0.774	0.253	0.762	0.081
C20:4n-3	0.110	0.029	0.318	0.066	0.110	0.030	0.185	0.050
C20:2n-6	0.480	0.112	0.279	0.042	0.469	0.117	0.284	0.029
C20:1n-11	0.061	0.017	0.060	0.009	0.060	0.016	0.055	0.007
C20:1n-9	0.303	0.045	0.221	0.040	0.286	0.050	0.256	0.011
C20:1n-7	0.162	0.022	0.178	0.021	0.197	0.040	0.156	0.026
C20:0	0.059	0.004	0.051	0.003	0.059	0.009	0.045	0.005
C21:5n-3	0.005	0.002	0.228	0.037	0.008	0.006	0.123	0.044
C22:5n-6	0.252	0.120	0.042	0.008	0.200	0.065	0.038	0.015
C22:6n-3	**3.44**	**0.572**	**9.66**	**1.04**	**3.041**	**0.637**	**6.77**	**1.50**
C22:4n-6	**0.701**	**0.257**	**0.143**	**0.026**	**0.551**	**0.130**	**0.192**	**0.038**
C22:5n-3	0.857	0.360	4.52	0.556	0.760	0.250	2.69	1.01
C22:2n-6	0.013	0.002	0.010	0.002	0.012	0.002	0.010	0.002
C22:1n-11	0.002	0.000	0.001	0.000	0.002	0.000	0.002	0.001
C22:1n-9	0.030	0.006	0.035	0.004	0.031	0.007	0.028	0,004
C22:1n-7	0.009	0.001	0.014	0.002	0.012	0.002	0.011	0,002
C22:0	0.077	0.011	0.067	0.015	0.081	0.021	0.050	0.008
C23:0	0.061	0.011	0.050	0.013	0.057	0.015	0.036	0.007
C24:1n-9	0.054	0.008	0.081	0.021	0.064	0.019	0.061	0.013
C24:0	0.190	0.027	0.189	0.045	0.209	0.048	0.138	0.023

Values are presented as means ±SD of wt%. HF, high fat; KO, krill oil; KH, krill hydrolysate; PRI, pristine; SFA, saturated fatty acids; FA, fatty acids; MUFA, monounsaturated fatty acids; PUFA n-3/-6, omega-3/-6 polyunsaturated fatty acid

Table 3.3: Double sided p-value t-test of fatty acid composition (g fatty acids/100 g total fatty acids) in plasma and liver after 8 weeks of dietary intervention.

PLASMA				**Liver**		
	HF vs KO	**HF vs KH**	**HF vs PRI**	**HF vs KO**	**HF vs KH**	**HF vs PRI**
C10:0	0.256	0.849	0.366	0.068	0.498	0.235
C12:0	0.073	0.250	0.241	0.002	0.389	0.067
C14:1n-5	0.016	0.208	0.030	0.028	0.350	0.326
C14:0	0.040	0.228	0.009	0.252	0.393	0.031
C15:0	0.006	0.370	0.055	0.632	0.298	0.005
C16:1n-9	0.915	0.405	0.083	0.186	0.362	0.200
C16:1n-7	0.225	0.934	0.001	0.515	0.845	0.009
C16:1n-7t	0.149	0.791	0.010	0.259	0.225	0.409
C16:1	0.000	0.263	0.000	0.114	0.460	0.002
C16:0	0.000	0.877	0.003	0.851	0.438	0.006
C17:0	0.793	0.600	0.207	0.203	0.688	0.186
C18:3n-6	0.000	0.672	0.000	0.000	0.017	0.000
C18:4n-3	0.001	0.325	0.000	0.001	0.274	0.064
C18:2n-6	0.181	0.164	0.390	0.000	0.672	0.000
C18:3n-3	0.286	0.097	0.506	0.187	0.669	0.443
C18:1n-9	0.951	0.759	0.014	0.036	0.481	0.186
C18:1n-7	0.054	0.521	0.004	0.086	0.610	0.095
C18:1t	0.252	0.063	0.984	0.006	0.921	0.000
C18:1	0.006	0.001	0.000	0.035	0.014	0.903
C18:0	0.089	0.446	0.013	0.894	0.263	0.007
C20:4n-6	0.000	0.175	0.000	0.000	0.783	0.000
C20:5n-3	**0.000**	**0.520**	**0.000**	**0.000**	**0.817**	**0.000**
C20:3n-9	0.000	0.205	0.000	0.004	0.727	0.002
C20:3n-6	0.003	0.453	0.002	0.188	0.787	0.815
C20:4n-3	0.000	0.362	0.000	0.000	0.991	0.003
C20:2n-6	0.000	0.713	0.003	0.000	0.864	0.000
C20:1n-11	0.780	0.535	0.437	0.952	0.941	0.409
C20:1n-9	0.283	0.809	0.440	0.002	0.483	0.011
C20:1n-7	0.116	0.004	0.878	0.161	0.047	0.608
C20:0	0.424	0.166	0.489	0.000	0.956	0.000
C21:5n-3	0.000	0.890	0.000	0.000	0.213	0.000
C22:5n-6	0.000	0.221	0.000	0.000	0.298	0.000
C22:6n-3	**0.000**	**0.079**	**0.000**	**0.000**	**0.212**	**0.000**
C22:4n-6	0.000	0.065	0.000	0.000	0.164	0.000
C22:5n-3	0.000	0.119	0.000	0.000	0.538	0.000
C22:2n-6	0.872	0.036	0.478	0.001	0.435	0.002
C22:1n-11	0.541	0.780	0.042	0.000	0.441	0.173
C22:1n-9	0.000	0.014	0.000	0.097	0.631	0.575

C22:1n-7	0.000	0.007	0.001	0.000	0.003	0.020
C22:0	0.371	0.699	0.017	0.157	0.581	0.000
C23:0	0.585	0.095	0.009	0.073	0.533	0.000
C24:1n-9	0.001	0.249	0.021	0.004	0.223	0.237
C24:0	0.485	0.941	0.187	0.939	0.341	0.001

Values are presented as means ±SD of wt%. HF, high fat; KO, krill oil; KH, krill hydrolysate; PRI, pristine; SFA, saturated fatty acids; FA, fatty acids; MUFA, monounsaturated fatty acids; PUFA n-3/-6, omega-3/-6 polyunsaturated fatty acids.

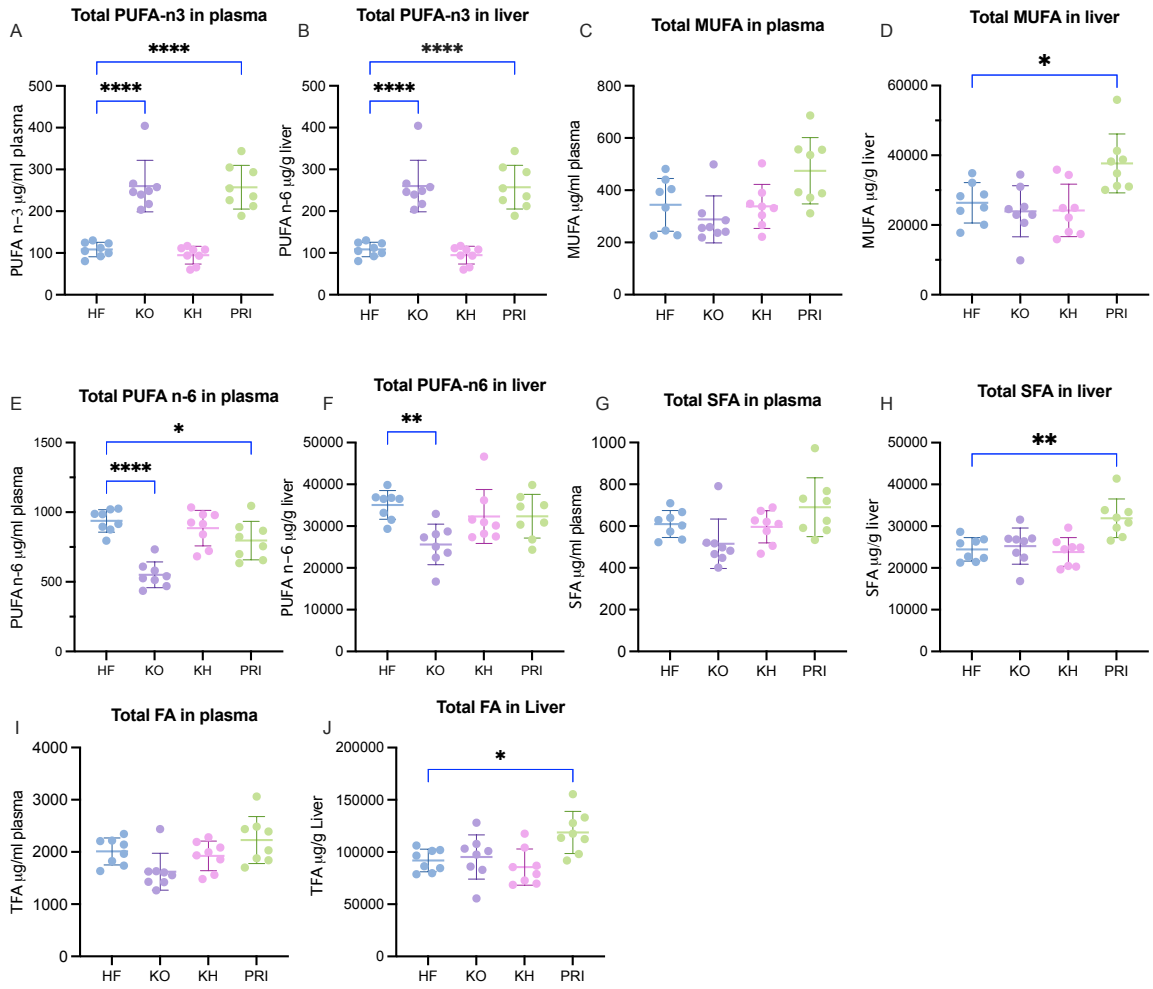

Figure 3.7 A-J: Fatty acid concentration in plasma and liver of (n=8) rats after 8 weeks of diet. The values presented are absolute concentration of main FA classes per µg/ml plasma and µg/g liver. The data were analyzed in GraphPad prism. One-way ANOVA with Dunnet's multiple comparisons test was used to determine statistical significance difference between control (HF) and the groups. Statistical significance difference compared to control (HF) are indicated with asterisks: * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001. Abbreviation: PUFA-n3/ -6,

omega-3/ -6 polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid; FA, fatty acid; HF, high fat diet; KO, krill oil; KH, krill hydrolysate; PRI, pristine.

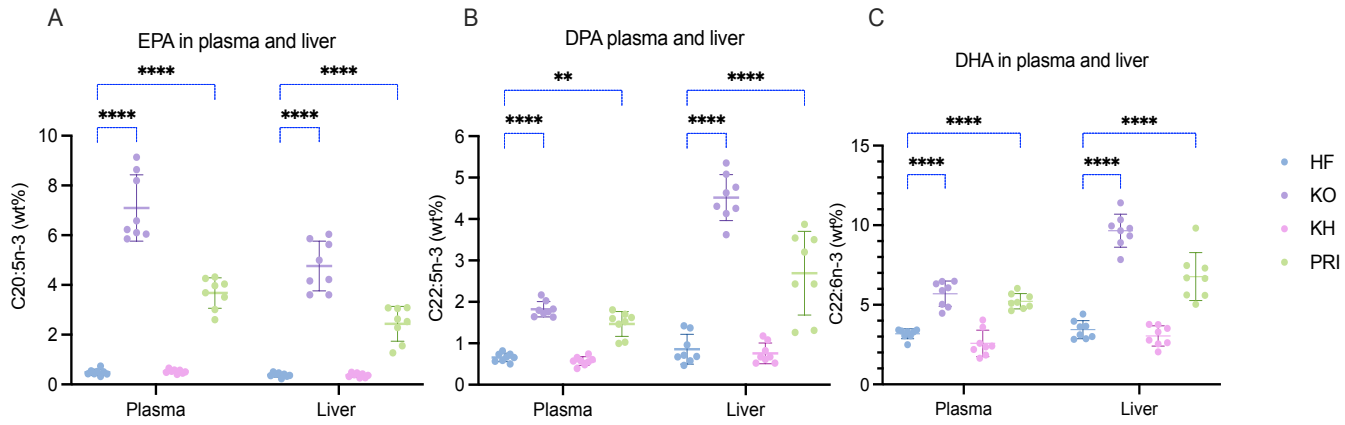

Figure 3.8 A-D: Specific omega-3 polyunsaturated fatty acids composition in plasma and liver (n=8) after 8 weeks of diet measured as weight % of total FAs in plasma and liver. A) EPA composition in plasma and liver. B) DPA composition in plasma and liver. C) DHA composition in plasma and liver. D) HPA composition in plasma and liver. The data were analyzed in GraphPad prism. Statistical significance difference compared to control (HF) are demonstrated with asterisks: ** P<0.01, *** P<0.001 and **** P<0.0001. Abbreviation: EPA; eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; HF, high fat diet; KO, krill oil; KH, krill hydrolysate; PRI, pristine.

DISCUSSION

This thesis focus was to study the effect of krill and krill hydrolysate diet, separately and in combination (as "Pristine") in male Wistar rats together with a high-fat (HF) "Western" diet as a background diet. The effect on body weights, weight of fat tissue, liver, kidney, and heart as well as plasma glucose and lipids were studied. Additionally, plasma and liver FAs were studied to observe the uptake of the different FAs in the diets.

MAIN FINDINGS

The overall findings in this study, were increased body- and organ weight as well as an increase in total liver lipids, when the rats consumed pristine diet together with the HF background diet. Notably, compared to a LF control diet, the HF control diet did not result in total weight gain or increased weight of adipose tissues. Based on the present experiment, we cannot know if pristine would have had the weight-increasing effect if the rats were given pristine together with a LF diet instead. As there might have been an interaction between the HF diet and pristine to increase body weight, which might not have been seen with a LF diet. In any case, pristine was hypothesized to counteract any body weight increase on HF diet, but the results showed the opposite effect, with highly consistent data for total body weight as well as the different adipose tissues. On the other hand, administering krill oil and krill hydrolysate individually with HF background diet did not have any marked effect on body fat thereby, indicating an interplay between these two nutritional components of krill when combined rather than an additive effect. Taken together, our data do not show any benefits of consuming krill, krill hydrolysate or pristine, but rather a weight-increasing effect of pristine.

Diet is one of the critical environmental factors for the development of obesity. Increased intake of fat and energy-dense diet is associated with body weight related to obesity and metabolic diseases (73). Previous studies have reported that HF diets promote weight gain and increased risk of CVD (76). The increase in weight gain in the present study could be due to the rats overeating the feed and/or having low energy expenditure. Moreover, the type of fat consumed has a vital impact on health in addition to the amount of fat (76). KO is a good source of polyunsaturated fatty acids with high bioavailability (77). In a previous study of krill oil and krill protein supplementation together with a HF Western diet done on Apo-E deficient mice, characterized by hyperlipidemia and susceptibility to atherosclerosis, no difference was observed in body weights of the mice (78). When taking our results and the Apo-E deficient

mice study into account, the data suggest that substituting most of the soybean oil (used in the HF feed) for krill oil does neither has a weight decreasing or increasing effect. Moreover, previous studies have reported metabolic health benefits of food sources with high polyunsaturated fatty acids, including reduced risk of CVD (76). To our surprise, we found no such evidence despite the high intake of marine omega-3 PUFA in the present rat experiment. In the Apo-E deficient mice study (78), liver weight of the rats that received krill oil treatment was significantly lower than HF-control, while liver weight of KO+krill protein and of krill protein alone was not different from control.

HF supplemented with pristine significantly increased mesenteric, perirenal and epididymal WAT compared to control HF-diet alone. In contrast to liver weight, these results are inconsistent with the findings in Apo-E mice, where no significance difference was observed in adipose tissue weight among intervention groups (78). It is possible that our findings reflect that the animals fed pristine consumed more feed, but this is however speculative since we did obtain complete data on the amount of diet consumed, and the individual feed intake of each rat was nonetheless difficult to assess because four and four rats were caged together.

While a significant increase in WAT was observed in rats fed with HF diet and pristine when compared HF control, no increase in interscapular BAT (iBAT) was discovered. Thus, visceral adipose tissue depots (mesenteric, perirenal, and epididymal adipose tissue) increased, while no beneficial effects on BAT were observed. Excess visceral adipose tissue is associated with several negative health effect including type 2 diabetes (79), insulin resistance (80) and inflammatory diseases (81). However, when only krill oil or krill hydrolysate supplemented with HF-diet, mWAT, pWAT and eWAT were not affected negatively. While our data do not support that krill oil or krill hydrolysate have a positive effect on visceral fat storage, i.e. by helping to reduce fat storage in the abdomen, our data at least suggest that krill oil or krill hydrolysate alone do not contribute to increase abdominal fat.

Despite the increased body weight and fat mass, dietary supplementation with pristine and HF were not associated with any significant effect on plasma lipid levels. Similarly, KO and KH supplementation had no impact on plasma lipid levels when compared to HF-diet. In contrast our findings, a previous human study that compared fish oil to krill oil supplementation, reported that there is a lowering effect of KO supplement on TAG and total cholesterol (82). Increase in plasma lipids of LDL and TAG are associated with adverse health effects, in

particular CVD (2,39). Although increases in LDL and TAG were insignificant in this study, it is unknown what kind of effect a HF-diet supplemented with pristine will have on these rats over a longer period of time. The experiment conducted in this study lasted for only 8 weeks. We cannot rule out that a significant increase in LDL and TAG levels in the plasma would have been observed if the experiment had lasted longer than 8 weeks, although the increased fat mass in the pristine-supplemented rats might have counteracted such a possible beneficial effect on blood lipids.

No significant changes in plasma glucose and cholesterol levels were discovered in this study when HF-diet was supplemented with only krill oil or krill hydrolysate or both. Three studies studying the effect of krill oil of plasma cholesterol reported that krill oil reduced cholesterol plasma levels (83–85). However, a network meta-analysis on krill oil versus fish oil did not find any significant cholesterol-modifying effect of krill oil (86). It should be noted that the reports from (83–85) were conducted on rats, humans, and mice respectively. Also, the study in the rats reported increased plasma cholesterol and glucose levels with phospholipid-type krill oil, but not for whole-type krill oil (83). Thus, a possible explanation for our result could be that the krill oil we used was mostly composed of whole krill oil. Another possible cause could be that krill oil was supplemented together with HF diet which was not done in the other studies.

Plasma and liver concentrations of omega-3 PUFAs (DHA, EPA and DPA) increased when HF supplementing the rats with only KO or pristine, as expected as KO is an abundant source of these long-chain omega-3 PUFAs (60). The increase in plasma and liver levels of omega-3 PUFAs observed in this study is expected to have several health benefits, including a beneficial alteration of hepatic genes expression, enhancement of fatty acid oxidation, lowered inflammation and improves insulin sensitivity (73,82). Furthermore, a study conducted by Qi et al.,(87) demonstrated that diets rich in omega-3 PUFAs had a lowering effect on TAG concentrations in mice via reducing endogenous TAG synthesis (73). Thus, it is surprising that supplementation of KO together with the HF diet did not improve any measured components of metabolic health in the rats. The reasons for this lack of effect are unclear.

Interestingly, in contrast to omega-3 PUFAs, there was a significant decrease in plasma omega-6 PUFA content when HF-diet was supplemented with KO and pristine compared to HF control, while in the liver, the concentration of omega-6 PUFA was significantly reduced with KO supplementation. A previous study conducted on mice reported that diets rich in KO increased total omega-6 PUFA but simultaneously decreased omega-6 PUFA proportions in both plasma and liver, and therefore improved the omega-3 PUFA/omega-6 PUFA ratio (88). Therefore, these results are congruent with our findings showing an increased omega-3/omega-6 PUFA ratio. Both SFA and total FAs had higher plasma and liver levels when HF diet was supplemented with pristine. Some studies have shown that SFAs may contribute to metabolic disorders because of its association with lower insulin sensitivity and increased atherosclerotic parameters (39). We also observed no significant increase in plasma total MUFA content but a significant increase of MUFA levels in the liver in pristine-supplemented rats. A similar study conducted in mice also reported no increase in plasma MUFA content, but a decrease in hepatic MUFA (88). Again, these different findings illustrate the challenge of interpreting data from different experimental studies in different strains of mice and rats and warrants cautious interpretation of the generality of our findings.

In conclusion, it is important that, as was expected, plasma and liver concentrations of EPA, DHA, and DPA were significantly higher in the rats that consumed krill oil and pristine. Krill consists of phospholipids and TAG with phosphatidylcholine as the primary phospholipid, and omega-3 PUFA seen in krill oil have high bioavailability (82). Yet, we found no beneficial metabolic effect of krill oil, alone or together with krill protein hydrolysate as a component of "pristine", but rather a weight- and fat mass-increasing effect of KO when supplemented together with krill hydrolysate, at least in HF diet background and the Wistar rats that were used in the present study.

METHOD DISCUSSION

In this study, the animals were fed different types of diets that were designed to contain an equal amount of energy but were of different sources. The animals had controlled access to the diets and the diets were given on specific days in the week to establish a routine in the rats. Furthermore, the cages were changed and cleaned once a week to maintain hygiene while not excessively disturbing and inducing stress in the animals.

To minimize the risk of selection bias in the current study, a randomizer (89) was used when distributing the rats to the cages and then the cages were randomized to diet, with two cages per diet. The rats were also euthanized in the same order as they started the diet, thus ensuring the same length of diet intervention. By using proper randomization methods during assignment to groups and cages, it is ensured that each experimental unit has an equal probability of getting a specific treatment and that human judgement or interference (consciously or subconsciously) does not introduce bias that can affect the outcome (90).

For the results in this study, statistical analysis was performed using ANOVA with Tukey's multiple comparison test to compare the diet groups with each other. When comparing the intervention groups to HF control, Dunnett's multiple comparisons test was used. The effect of HF diet was compared to that of LF diet using unpaired two-tailed *t*-test, as this was a focused sub-analysis to test the differences between two dietary backgrounds.

In any study of dietary intervention, it is crucial to control feed intake as much as possible. However, having exact control of the feed intake of the individual rat can be challenging. In our study, for practical reasons, eight rats in a diet group were divided into two groups of four that resided in one cage each. Thus, we cannot know how much each rat ate. This likely introduced variation in the measurements and gave inaccurate data for food intake. Nevertheless, we cannot rule out that the increased body weight seen with Pristine (KO+KH) may have occurred because the rats on average ate more. This was observed already in the first week of the animal experiment as the feeding frequency was increased from two times a week to three times a week, because the rats in this group had eaten somewhat more than was expected. Moreover, feed remnants in each cage were only measured in week 0 and week 4, thereby limiting the data to calculate weight gained per energy ingested ("feed efficiency") per cage let alone per individual rat.

CONCLUSION

In this study, after 8 weeks of dietary intervention, the results were not as expected. There were no benefits observed in body- and organ weight in the rats that were supplemented with krill oil and krill hydrolysate alone with a high fat background diet when compared to those that consumed HF diet. Moreover, no benefits in body- and organ weight were detected in the rats that consumed pristine with a high fat diet when compared to HF, rather a negative effect on weight. Additionally, pristine had a weight increasing effect on the different white adipose tissue depots. Furthermore, no marked effect was observed in plasma lipid levels. Therefore, these findings suggest that pristine has no counteracting/anti-obesity benefits on the rats when consumed with a HF-Western diet, but rather a weight-inducing effect.

FUTURE PERSPECTIVES

In this study, only few metabolic health parameters were investigated. However, more extensive analysis, such as effect on gene expression involved in lipid metabolism and enzyme activities must be done to conclude the metabolic health effect of krill oil and krill hydrolysate and its combination as pristine. To further investigate the impact on body weight, measurement of food intake should be explored. This will likely remove the variation that was introduced in the measurements. Additionally, longer intervention period could be necessary to be able to observe the long-term effect on plasma lipids. We suggest that a longer intervention period should be carried out with this same experiment as we have doubts that the no significant effect of our dietary intervention on plasma lipids could be as a result of a short period experiment.

While particular attention has been paid to the lipid content (krill oil) in krill studies, less have been to elucidate on the effect of the protein content in krill and their combination in both animal and human studies. It is also necessary to document the beneficial effect of KO with more human studies and to elucidate if these effects differ from this after regular fish and FO intake.

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