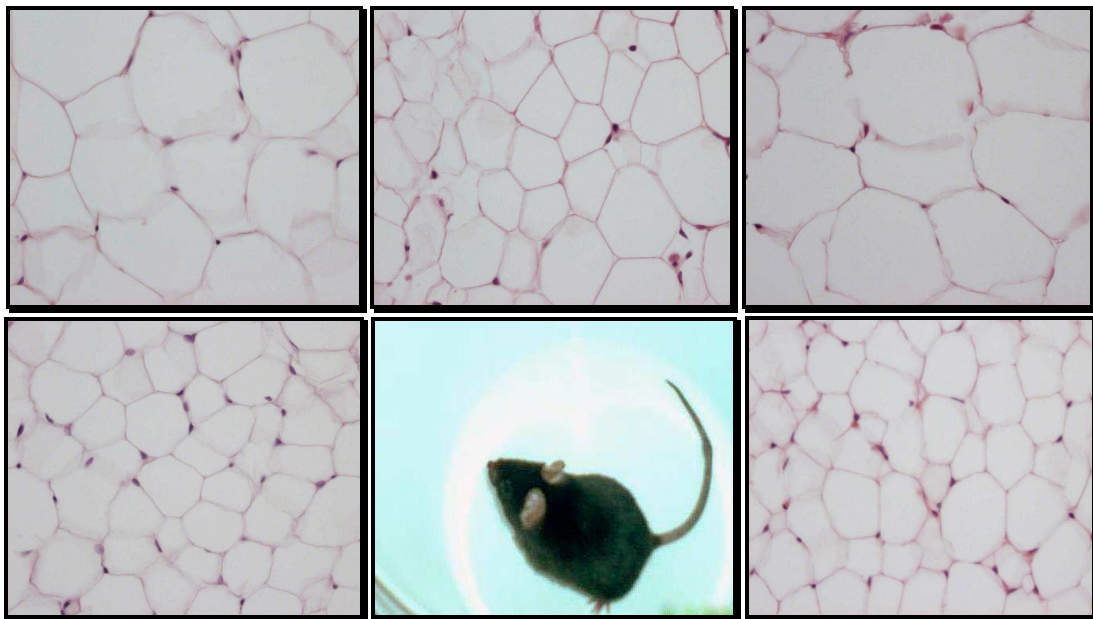


**THE ROLE OF DIETARY FAT AND CARBOHYDRATES
IN DEVELOPMENT OF ADIPOSE TISSUE
INFLAMMATION**



HA THI NGO

MASTER THESIS IN HUMAN NUTRITION

INSTITUTE OF MEDICINE, UNIVERSITY OF BERGEN (UIB)

NATIONAL INSTITUTE OF NUTRITION AND SEAFOOD RESEARCH (NIFES)

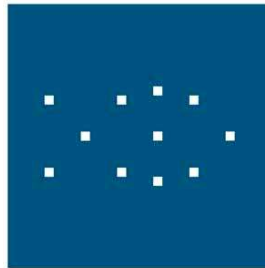
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Cover illustration: *In the center; private photograph taken of a CB57BL/6J mouse from the feeding-experiment. From the bottom left clockwise; histology pictures(400X, scale bar =50µm) of paraffin-embedded and H&E stained sections of inguinal white adipose tissue (iWAT) from mouse fed: fish oil + protein, fish oil + sucrose, low energy, corn oil + protein and corn oil + sucrose.*

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Bergen, June 2009

Ha Thi Ngo

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LIST OF ABBREVIATIONS

AA	Arachidonic acid (20:4n-6)
ALA	Alpha- linolenic acid (18:3n-3)
ANOVA	Analysis of variance
ATM	Adipose tissue macrophage
ATP	Adenosine triphosphate
bHLH-Zip	Basic-helix-loop-helixleucine zipper
BMI	Body mass index
CCL2	Chemokine (C-C motif) ligand 2
CD68	Cluster of differentiation 68
cDNA	Complementary deoxyribonucleic acid
CLS	Crown like structures
COX	Cyclooxygenase
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
eWAT	Epididymal white adipose tissue
GLC	Gas liquid chromatography
iBAT	Interscapular brown adipose tissue
IKK	Inhibitor of kappa B kinase
IL-6	Interleukin 6
IR	Insulin resistance
iWAT	Inguinal white adipose tissue
JNK	c-Jun N-terminal kinase
LA	Linoleic acid (18:2n-6)
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene

MCP-1	Monocyte chemoattractant protein-1
MUFA	Monounsaturated fatty acid
n-3	Omega-3
n-6	Omega-6
NF- κ B	Nuclear Factor kappa B
PAI-1	Plasminogen activator inhibitor-1
PCR	Polymerase chain reaction
PG	Prostaglandin
PPAR- γ	Peroxisome proliferator- activated receptor gamma
Pref-1	Preadipocyte factor-1
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
RIN	RNA integrity number
RNA	Ribonucleic acid
RT	Reverse transcriptase
rWAT	Retroperitoneal white adipose tissue
SAA	Serum amyloid A
SFA	Saturated fatty acid
SCD1	Stearoyl-Coenzyme A desaturase 1
SEM	Standard error of the mean
SP3	Service pack 3
SREBP-1c	Sterol regulatory element binding protein-1c
STAMP2	Six- transmembrane protein of prostate 2
SVF	Stromal vascular fraction
TBP	TATA box binding protein
TLR4	Toll-like receptor 4
TNF- α	Tumor necrosis factor alpha
TNF1	Tumor necrosis factor receptor 1
TX	Thromboxane
UCP-1	Uncoupling protein 1
US, USA	United States of America
VBA	Visual Basic Applications
WHO	World Health Organization

ABSTRACT

Obesity is considered as a chronic low-grade inflammatory condition, which can be characterized by increased levels of the proinflammatory cytokines such as TNF- α and IL-6, and macrophage infiltration in adipose tissue. Previous studies have reported that fish oil enriched in the polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) can protect against inflammation in rodents and humans. The aim of this study was to find out whether the type of dietary fat can modulate inflammation. In addition, investigate if the carbohydrate to protein ratio could influence the development of obesity and the inflammatory response.

Thirty five male C57BL/6J mice were divided into 5 different groups (n=6) and fed 5 different diets *ad libitum*. The diets given were; low energy, fish oil + protein, fish oil + sucrose, corn oil + protein, and corn oil + sucrose. Body weight was recorded weekly and feed intake was recorded 3 times a week. The mice were terminated after 10 weeks of feeding, and adipose tissues and blood samples were collected. Gene expression analysis was performed with quantitative real-time PCR (qPCR). Moreover, paraffin-embedded sections of adipose tissue were stained with H&E and examined with a microscope. In addition, plasma parameters were analyzed along with the determination of fatty acid compositions in RBC, liver and adipose tissue. The different methods were performed in order to assess the development of obesity and inflammation, in adipose tissue from mice fed the different diets.

The results showed that the anti-inflammatory effect of fish oil was abolished in combination with sucrose in the diet. The fish oil + sucrose group showed significantly higher expression levels of the inflammatory cytokines TNF- α and IL-6, including the macrophage markers F4/80 and CD68 compared to the other treatment groups. Increased adipose tissue mass and hypertrophied adipocytes are presumed to be due to the high-sucrose enrichment in the diets. Sucrose is also considered to be the adipogenic factor, because of its ability to direct fat on to adipose tissue, and attenuate the known positive effects of fish oil.



1 INTRODUCTION

1.1 HUMAN OBESITY

Obesity is a condition where excess body fat has accumulated, which can lead to multiple organ-specific pathological consequences (Haslam, Sattar *et al.*, 2006). The condition is commonly defined by body mass index (BMI), where the weight in kilograms is divided by the square of the height in metres (kg/m^2). The World Health Organization (WHO) classifies a BMI of $25.0 \text{ kg}/\text{m}^2$ or higher as abnormal, and a BMI of $30 \text{ kg}/\text{m}^2$ or higher as obese in adults. The normal range of BMI is set between 18.50 - $24.99 \text{ kg}/\text{m}^2$ (WHO, 2000). A better measure of metabolic risk than BMI is waist circumference. This measure is more directly proportional to total body fat and the amount of metabolically active visceral fat (Haslam, Sattar *et al.*, 2006; Haslam and James, 2005).

1.1.1 *The prevalence of obesity*

Excess body fat is now considered to be the sixth most critical risk factor contributing to the increased risk of many diseases worldwide (Haslam and James, 2005). Approximately 9.8 % (9.6-10.0 %) of the world's adult population were obese in 2005, with the estimated total numbers of 396 million (388-405 million) people (Kelly, Yang *et al.*, 2008). Figure 1.1 shows the average regional prevalence of obesity for both men and women with different ages in the world. The estimates are based on measured BMI in appropriate population samples (Haslam and James, 2005). The prevalence of obesity in developed countries is rapidly increasing, but the US is still in the leading position with 32.2 % of adult being obese in 2003-2004 (Flegal, Carroll *et al.*, 2002; Ogden, Carroll *et al.*, 2006).

The prevalence of obesity for adults in Norway in 2000-2003 was around 19.5 % for men and 20 % for women. The Norwegian citizens in Finnmark county had the highest prevalence of obesity, whereas the lowest were reported in the capital, Oslo (Ulset, Undheim *et al.*, 2007). Norwegian studies also report that children should be focused on as a target group in order to combat adulthood obesity (Dvergsnes and Skeie, 2009; Handeland and Hjelmsaeth, 2009).

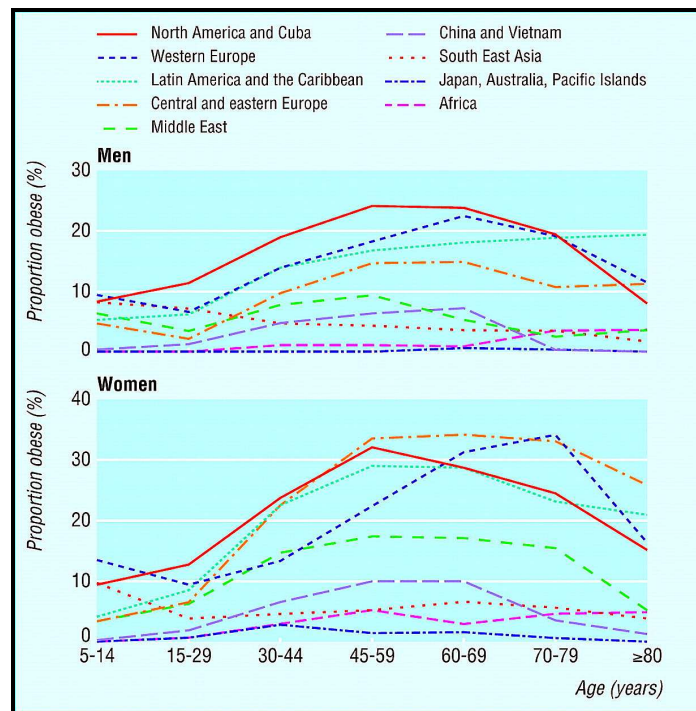


Figure 1.1: Prevalence of obesity worldwide, from Haslam *et al.* (2006), *BMJ* 333, 640-2.

1.1.2 Obesity – causative factors

The rapid increase of the prevalence of obesity in the world is most likely caused by an energy imbalance. More calories are consumed than expended. Which factors might be the cause of this energy imbalance? The changing lifestyle of modern people, where the caloric consumption and portion size has increased along with daily intake of sugar-sweetened beverages, will contribute to such imbalance. Secondly, fast food and other meals outside the home, packed with unhealthy fat and refined carbohydrates will also contribute to the energy imbalance. The media works as a powerful source with constant advertising and promoting the overconsumption of such foods, especially among children (Wiecha, Peterson *et al.*, 2006). Another factor that promotes reduced energy expenditure is the more sedentary lifestyle, such as increased television and computer use. The environment at home, school or work might require less walking, or less physical education and overall activity. Vehicles, internet connections and remote controls also contribute to the reduced activity. The physical activity requirements in a daily life have been strongly reduced for most citizens in the western countries during the last decades (Malterud and Tonstad, 2009).

The changing lifestyle with increased energy intake rather than energy expenditure is considered as main causative factors of obesity. However, genetic factors can also affect the prevalence of obesity on many levels, such as; portioning of energy metabolism, control of food intake and culminating in a state of energy imbalance (Cecil, Watt *et al.*, 2006). A study by De Castro (1999) observed twins in their natural environment, where strong influences of heredity were found on the total amounts of food energy, macronutrients and fluid intake. The amount of energy ingested were not only influenced by anatomy and physiology of the individual, but other mechanisms such as the psychology and sociology of the individual were of significance (de Castro, 1999). Family studies along with twin familial aggregation have brought evidence of heritable connection between human obesity and genes (Cecil, Watt *et al.*, 2006).

Furthermore, the result of an unhealthy lifestyle with nutrient overload causing energy imbalance and obesity is among other factors the storage of excess energy in adipocytes (de Ferranti and Mozaffarian, 2008). Obesity can also lead to an array of associated metabolic disorders including increased risk of insulin resistance, fatty liver disease, atherosclerosis, type 2 diabetes, cardiovascular disease, airway disease and some types of cancers. This cluster of pathologies now constitutes the largest global health threat (Heilbronn and Campbell, 2008; Hotamisligil, 2006; Wellen, Fucho *et al.*, 2007).

1.1.3 Obesity – low-grade inflammatory condition

Obesity was first considered as a chronic low-grade inflammatory condition when Hotamisligil *et.al.* (1993) described an increased level of the proinflammatory molecule tumor necrosis factor –alpha (TNF- α), after diet induced obesity in rodents and demonstrated its effect on insulin sensitivity (Hotamisligil, Shargill *et al.*, 1993). Since then, several studies have confirmed the association between an increase in adipose tissue mass and increased levels of other inflammatory molecules in addition to TNF- α . These circulating proinflammatory molecules are; plasminogen activated inhibitor (PAI-1), interleukin (IL)-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) (Clement, Viguerie *et al.*, 2004; Lumeng, Deyoung *et al.*, 2007). More information about these proinflammatory molecules will be presented in section 1.2.2.

1.1.4 Insulin resistance

Insulin resistance (IR) is a key factor linking obesity to type 2 diabetes (Hotamisligil, Arner *et al.*, 1995; Qatanani, Szwegold *et al.*, 2009). The insulin's inability to act normally in regulating nutrient metabolism in peripheral tissues leads to insulin resistance (Xu, Barnes *et al.*, 2003). IR is also part of the metabolic syndrome and believed to play a central role in other pathological conditions associated with obesity such as; dyslipidemias, atherosclerosis, hypertension and cardiovascular diseases (Hotamisligil, Arner *et al.*, 1995). Insulin is a hormone with multiple functions, but its main function is to stimulate glucose uptake into insulin-sensitive tissues after a meal and regulate energy homeostasis (Melancon, Bachelard *et al.*, 2006). The insulin-sensitive tissues include the muscle, liver and adipose tissue (Hotamisligil, 1999). Insulin inhibits lipolysis and stimulates *de novo* fatty acid synthesis in adipocytes (Laviola, Perrini *et al.*, 2006). Increased level of free fatty acids (FFA) due to lipolysis are observed in obesity and type 2 diabetes (Kahn, Hull *et al.*, 2006). Furthermore, an impaired glucose transport in skeletal muscle cells leads to an overall decreased glucose uptake in these cells. While IR in the liver leads to an increased hepatic glucose production, as a result of insulin failing to suppress the gluconeogenesis (de Luca and Olefsky, 2006; de Luca and Olefsky, 2008).

Obesity as a result of overnutrition can lead to adipocyte hypertrophy, due to the accumulation of lipids in adipose tissue. The inflammatory process continues with accumulation of macrophages in the adipose tissue. Further initiation of the inflammatory process comes from the increased production and secretion of proinflammatory cytokines and chemokines from the adipocytes (Shoelson, Herrero *et al.*, 2007). These secreted molecules can act in a paracrine or autocrine manner to augment the proinflammatory state and cause localized insulin resistance. The stress and inflammatory signals along with the factors derived from adipose tissue, can also lead to local insulin resistance in both liver and muscle (figure 1.2). (de Luca and Olefsky, 2006).

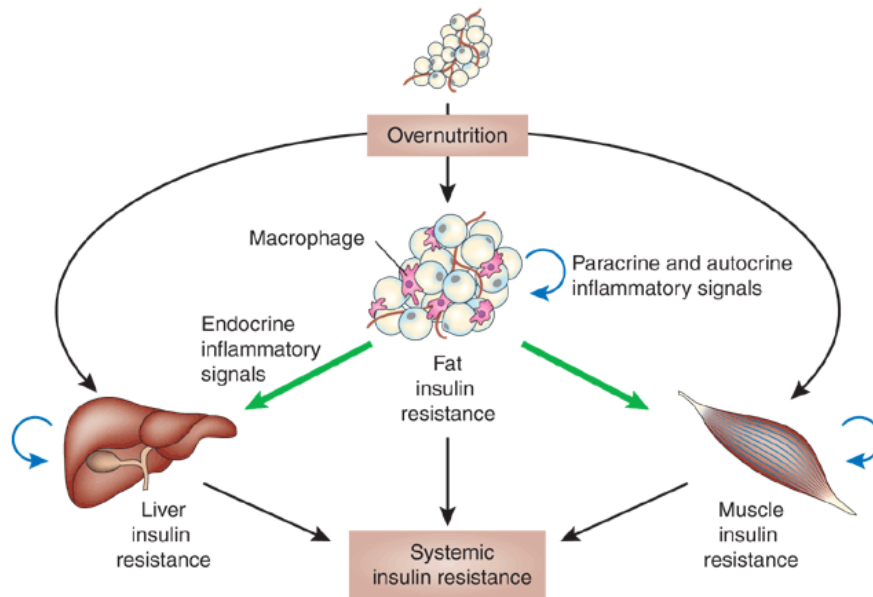


Figure 1.2: The link between obesity-induced inflammation and systemic insulin resistance. From de Luca and Olefsky, (2006).

1.1.5 Metabolism

The objective with this section is not to give an overview of the metabolism in obesity, but to present two genes that play a central role in the lipid metabolism, that again can be linked to obesity development.

STEROL REGULATORY ELEMENT-BINDING PROTEIN

The three sterol regulatory element-binding protein (SREBPs) isoforms; SREBP-1a, SREBP-1c and SREBP-2 are recognized to play a central role in the regulation of cholesterol and fatty acid homeostasis. SREBPs are part of the basic-helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors (Horton, 2002). SREBP-1c, mainly found in the liver is the major regulator of *de novo* lipogenesis and synthesis of triglycerides (Qin, Dalen et al., 2009). An upregulation of the SREBP-1c gene in the liver of animal models of obesity and type 2 diabetes increases the rate of fatty acid synthesis. The result is further aggravation of insulin resistance in these fatty liver diseases (Yang, Craddock et al., 2009). SREBP-1c may also play a role in the regulation of synthesis of polyunsaturated fatty acids (PUFA). The

enzymes Δ -5- desaturase and Δ -6-desaturase that perform the desaturation reactions are genes transcriptionally activated by SREBP-1 isoforms (Matsuzaka, Shimano et al., 2002).

A study by Liang *et al.* showed that SREBP-1c knockout mice fed a chow diet had normal body weights and liver weights compared to wild-type mice. However, further comparisons showed significantly increased levels of liver cholesterol, along with significantly reduced plasma cholesterol- and plasma triglycerides levels in the SREBP-1c knockout mice (Liang, Yang *et al.*, 2002).

STEAROYL-COENZYME A DESATURASE 1

The stearoyl- Coenzyme A desaturase (SCD) is a gene that converts saturated fatty acids into monounsaturated fatty acids (MUFA), mainly oleate (18:1) and palmitoleate (16:1) (Flowers and Ntambi, 2008; Ntambi, Miyazaki *et al.*, 2002). The converted MUFA are central components of triglycerides, cholesterol esters, and phospholipids (Jiang, Li *et al.*, 2005; Ntambi, Miyazaki *et al.*, 2002). One of the SCD isoforms found in mouse is SCD1, which is highly expressed in the liver, WAT and BAT (Jiang, Li *et al.*, 2005). An increased activity of SCD1 in cells has been suggested to influence the separation of fatty acids by promoting the fatty acid synthesis and decreasing the oxidation. Both dietary and hormonal factors such as glucose, fructose, saturated fatty acids and insulin can contribute to the elevation of SCD1 levels. Leptin and polyunsaturated fatty acids can on the other hand inhibit the elevation. Thus high levels of SCD1 may contribute to the development of obesity along with other chronic diseases (Flowers and Ntambi, 2009).

Previous studies report that SCD1 deficient mice are leaner than their wild-type counterparts and resistant to both diet-induced and genetically induced obesity (Jiang, Li *et al.*, 2005; Ntambi, Miyazaki *et al.*, 2002). The SCD1 deficient mice are also protected from cellular lipid accumulation and insulin resistance, due to reduced MUFA synthesis and increased lipid oxidation (Flowers and Ntambi, 2008; Flowers and Ntambi, 2009).

1.2 INFLAMMATION

Inflammation is the body's immediate response to infection or injury. The response is vital to initiate immunologic processes. This includes the elimination of invading pathogens and toxins, as well as repairing damaged tissue (Calder, 2006). As mentioned before, obesity may be considered as a chronic low-grade inflammatory state, as activated inflammatory pathways characterize adipose tissue in obese animals and humans (de Luca and Olefsky, 2008; Hotamisligil, 2006).

TOLL-LIKE RECEPTOR 4

The concept that obesity represents an inflammatory state introduces an aspect in terms of healthy and unhealthy adipose tissue. Dietary factors, fatty acids in particular, could potentially represent direct modulators of the inflammatory state as they are able to activate the Toll-like receptor 4 (TLR4) in adipose tissue (Shi, Kokoeva *et al.*, 2006). TLR4 is a part of the Toll-like receptor family whose function is to recognize bacterial and viral components and guard against microorganismal infections as part of the innate immune system (de Luca and Olefsky, 2008). TLR4 and its co-receptor CD14 (cluster of differentiation 14) binds to bacterial lipopolysaccharide (LPS) of gram-negative bacterial walls and mediates the inflammatory response (Medzhitov, 2001; Shi, Kokoeva *et al.*, 2006). LPS is able to activate both monocytes and macrophages, and stimulating them to produce proinflammatory cytokines such as TNF- α and IL-6 (Calder, 2002). Additionally, activation of the TLR4 complex results in a stimulation of the c-Jun amino terminal kinase (JNK) and the I κ B kinase- β (IKK β)/ nuclear factor- κ B (NF- κ B) pathways, by the mentioned proinflammatory cytokines; TNF- α and IL-6. The transcription factor, NF- κ B is considered a primary factor in the inflammatory response, where IKK- β is required for its activation (Shoelson, Lee *et al.*, 2003; Solt and May, 2008). Activation of these pathways results in upregulation of potential inflammatory mediators and subsequent insulin resistance (Cuschieri, Bulger *et al.*, 2008; Kahn, Hull *et al.*, 2006).

TLR4 negative mice are more obese than their wild-type littermates, but still have reduced inflammation and improved insulin resistance (Shi, Kokoeva *et al.*, 2006). Conversely, mice lacking six-transmembrane protein of prostate 2 (STAMP2), develop adipose tissue inflammation and spontaneous metabolic disease on a regular diet prior to becoming obese.

STAMP2 is designated as a metalloredutase which has the ability to coordinate inflammatory responses with metabolic function in adipocytes. The protein also play central role in the maintenance of systemic metabolic homeostasis (Wellen, Fucho *et al.*, 2007). The reports from these studies raise the suggestion that the size of adipose tissue and the degree of inflammation may not always be causally linked.

1.2.1 Macrophages

Macrophages are mononuclear phagocytes that play a central role in immunological and inflammatory processes. The function of macrophages in addition to providing an immediate defense against foreign organisms, is to eliminate dying cells (Heilbronn and Campbell, 2008; Lin, Faunce *et al.*, 2005). In a chronic inflammatory process locally secreted chemokines attract pro-inflammatory macrophages into the adipose tissue, where the formation of “crown-like” structures (CLS) takes place around enlarged dead or dying adipocytes (de Luca and Olefsky, 2008). The adipose tissue macrophages (ATMs) functions as a main source of proinflammatory cytokines; TNF- α and PAI-1 (Lumeng, Deyoung *et al.*, 2007). ATMs exacerbates the inflammation and insulin resistance by secreting these cytokines, which further activate the inflammatory process in adipocytes nearby (de Luca and Olefsky, 2008). Invasion of macrophages along with the secretion of proinflammatory immune mediators such as adipokines, cytokines, chemokines are associated with the increase of adipose tissue mass (Kempf, Rose *et al.*, 2006). Thus, it is also suggested that these macrophages are somewhat responsible for the development of type 2 diabetes in obese subjects (Lumeng, Deyoung *et al.*, 2007).

Several studies carried out report that macrophage infiltration in adipose tissue is characteristic for both obese rodents and humans (Cancello, Henegar *et al.*, 2005; Weisberg, McCann *et al.*, 2003; Xu, Barnes *et al.*, 2003). The infiltration of macrophages can either be recruited as a response to death of hypertrophied adipocytes (Cinti, Mitchell *et al.*, 2005), or by an increased monocyte chemoattractant protein-1 (MCP-1) expression in obesity induced mice adipose tissue (Kanda, Tateya *et al.*, 2006). More information about MCP-1 is presented in section 1.2.2.

MACROPHAGE MARKERS- CLUSTER OF DIFFERENTIATION 68 and F4/80

Cluster of differentiation 68 (CD68) is a glycosylated transmembrane protein, where high expression is especially found in macrophages and macrophage-related cells (Xu, Barnes *et al.*, 2003). By using a CD68 antibody which recognizes the antigen in immunohistochemical analysis, the macrophages are able to be detected in the tissue (Weisberg, McCann *et al.*, 2003).

The F4/80 monoclonal antibody is another macrophage marker frequently used in mouse tissue (van den Berg and Kraal, 2005). The antigen (Ag) glycoprotein F4/80 is considered to be one of the most specific cell-surface markers for murine macrophages (Lin, Faunce *et al.*, 2005). F4/80 is a member of the epidermal growth factor (EGF)-transmembrane 7 (TM7) family, but the function of F4/80 Ag is not completely understood (McKnight, Macfarlane *et al.*, 1996).

1.2.2 Cytokines and chemokines

Along with the increased levels of macrophage markers, can also elevated levels of cytokines be observed in adipose tissue inflammation. Cytokines are characterized as small proteins produced by a number of different cell types. They can act on almost every tissue and organs systems (Blok, Katan *et al.*, 1996). The cytokines designated as proinflammatory produced by monocytes and macrophages including adipose tissue are; IL-1 α β , IL-1 β , TNF- α and IL-6 (Simopoulos, 2002b). The most studied cytokines in association with obesity are TNF- α and IL-6, where elevated levels are shown in adipose tissue in obese subjects (Bastard, Maachi *et al.*, 2002; Cottam, Mattar *et al.*, 2004). These cytokines mediate the host's response to inflammation stimuli (Blok, Katan *et al.*, 1996).

TUMOR NECROSIS FACTOR-ALPHA

Tumor necrosis factor alpha (TNF- α) is a proinflammatory cytokine with many biological effects on lipid metabolism, coagulation and endothelial function. Stimulation of the NF- κ B IKK β is a result of activated TNF receptor (de Luca and Olefsky, 2008). As mentioned before, Hotamisligil *et al.* were the first one to show an increased level of TNF- α in obese rodents, and subsequently linking the TNF- α levels, obesity and insulin resistance in humans

(Hotamisligil, Arner *et al.*, 1995; Hotamisligil, Shargill *et al.*, 1993). Mice that are genetically deficient in TNF or the TNF receptor 1 gene (TNFR1) do not develop insulin resistance when fed a high-fat diet (Uysal, Wiesbrock *et al.*, 1997).

INTERLEUKIN- 6

Interleukins (IL) are proteins that mainly mediate the interactions between immune and inflammatory cells. ILs are able to promote cell growth along with differentiation and functional activation (Simopoulos, 2002b). IL-6 is a cytokine with multiple functions, where the ability to regulate the hepatic production of CRP is important (Bastard, Maachi *et al.*, 2006; Yudkin, Kumari *et al.*, 2000). Studies have reported that IL-6 may be involved in insulin resistance along with its complications (Bastard, Jardel *et al.*, 2000; Bastard, Maachi *et al.*, 2002). The ultimate stimulus for induction of IL-6 production from macrophages and adipocytes due to an obese state is poorly understood (Fantuzzi, 2005).

MONOCYTE CHEMOATTRACTANT PROTEIN-1

Monocyte chemoattractant protein-1 (MCP-1) also known as Chemokine (C-C motif) ligand 2 (CCL2) belongs to the CC chemokine family and function as a central ligand of chemokine receptor-2 CCR2 (Chen, Mumick *et al.*, 2005). MCP-1 is as mentioned before reported to play a key role in the recruitment of macrophages into the adipose tissue (Gustafson, Hammarstedt *et al.*, 2007). The result of the recruitment is an altered metabolic and endocrine activity of adipocyte cells, including insulin resistance (Dahlman, Kaaman *et al.*, 2005). On the other hand, a protection against insulin resistance has been observed in MCP-1 knockout mice fed a high-fat diet (Kanda, Tateya *et al.*, 2006).

1.3 ADIPOSE TISSUE

Which factors causing the inflammation process in obesity are still not completely understood, but the adipose tissue seems to play a central role in the relationship between obesity and chronic inflammation (Bluher, 2008). The adipose tissue is mainly divided into two types: white adipose tissue (WAT) and brown adipose tissue (BAT). In mammals, the adipose tissue consists of both WAT and BAT, but the WAT makes up the biggest majority of adipose tissue in the organism and functions as the storage of energy in the form of

triglycerides (Fantuzzi, 2005; Villena, Choi *et al.*, 2008). BAT on the other hand plays an important role in heat production through nonshivering thermogenesis (Yan, Burman *et al.*, 2006).

1.3.1 White adipose tissue (WAT)

WAT is a heterogeneous tissue consisting of mature adipocytes and cell types of various lineages in the stromal vascular fraction (SVF). This fraction is composed of pre-adipocytes, endothelial cells, fibroblasts, histiocytes, and macrophages (Heilbronn and Campbell, 2008). The WAT's function is not only storage of energy as fat, but it is also recognized to be an active endocrine organ, which has a high capacity to produce and secrete different adipokines, cytokines and chemokines (Clement, Viguier *et al.*, 2004; Heilbronn and Campbell, 2008). Some of the biologically active molecules secreted includes: leptin, adiponectin, resistin, TNF- α , IL-6, MCP-1, PAI-1, angiotensinogen, visfatin, retinol-binding protein-4, serum amyloid A (SAA) and others. WAT is a complex and active secretory organ which has the ability to both send and receive signals that modulate; energy expenditure, appetite, insulin sensitivity, inflammation and immunity (Shoelson, Herrero *et al.*, 2007).

The phenomenon of older mice secreting more cytokines from the adipose tissue was shown in a study by Wu *et al.* (2007). Data from mRNA expression levels of the proinflammatory cytokines among others; IL-6, TNF- α were significantly higher for the old mice compared to younger ones. The outcome for these data might be due to the role of macrophages and adipocytes in aged adipose tissue (Wu, Ren *et al.*, 2007).

1.3.2 Brown adipose tissue (BAT)

The BAT consists mainly of brown adipocytes, endothelial cells, interstitial cells, and preadipocytes which has the ability to both divide and differentiate new brown adipocytes. The BAT is in contrast to WAT packed with mitochondria, which makes it appear brown in colour. Uncoupling protein-1 (UCP-1) is a BAT-specific protein which carries proton or protons equivalents across the mitochondria without producing ATP. The result of this process is part of the energy stored in the proton gradient dissipates as heat (Gustafson, Hammarstedt *et al.*, 2007; Yan, Burman *et al.*, 2006).

1.3.3 Adipocyte differentiation

Adipogenesis is a process where undifferentiated precursor cells (preadipocytes) are differentiated into mature adipocytes (Gustafson, Hammarstedt *et al.*, 2007). Some of the genes involved in the adipocyte differentiation will be presented next.

PEROXISOME PROLIFERATOR- ACTIVATED RECEPTOR GAMMA

The peroxisome proliferators-activated receptor (PPAR) family consisting of the three isotypes: PPAR- α , PPAR- β / δ and PPAR- γ play an important role in the regulation of energy balance (Tsai and Maeda, 2005). There are two different isoforms of the ligand-activated transcription factor PPAR- γ ; PPAR- γ 1 expressed in non-adipose tissues, and PPAR- γ 2 which is mainly expressed in adipose tissue (Rangwala and Lazar, 2004; Stienstra, Duval *et al.*, 2007). In this thesis, the isoform PPAR- γ 2 will from now on be referred to as PPAR- γ . PPAR- γ plays a key role in the adipocyte differentiation, lipid metabolism and insulin sensitivity. Activation of PPAR- γ is carried out by dietary polyunsaturated fatty acids and fatty acid-derived molecules (Cecil, Watt *et al.*, 2006; Stienstra, Duval *et al.*, 2008).

Previous studies report that PPAR- γ deficient mice fed a high-fat diet are protected against adipocyte hypertrophy, obesity and insulin resistance (Jones, Barrick *et al.*, 2005; Kadowaki, Hara *et al.*, 2002). PPAR- γ ligands are able to reduce circulating levels of FFAs, and thus reverse insulin resistance (Rangwala and Lazar, 2004). It has also been reported that PPAR- γ has anti-inflammatory properties, by reversing macrophage infiltration and hence contributing to reduction of the pro-inflammatory gene expressions (Xu, Barnes *et al.*, 2003).

PREADIPOCYTE FACTOR-1

The adipocyte differentiation can also be regulated by the transmembrane protein preadipocyte factor-1 (Pref-1) (Lee, Villena *et al.*, 2003). Pref-1 belongs to the epidermal growth factor (EGF)-like super family, and is commonly found in preadipocytes (Barbu, Hedlund *et al.*, 2009; Sul, Smas *et al.*, 2000). One of the most important functions of Pref-1 is to inhibit the adipocyte differentiation (Barbu, Hedlund *et al.*, 2009; Villena, Choi *et al.*, 2008).

The important role of Pref-1 in adipocyte differentiation has been shown in mice lacking this gene, where the results were growth retardation along with skeletal abnormalities and obesity, when fed a high-fat diet (Moon, Smas *et al.*, 2002). On the other hand, an impaired adipocyte differentiation due to the inhibition of Pref-1 overexpression in transgenic mice, showed a remarkable reduction in adipose tissue mass. The expression of other adipocyte markers such as adiponectin was also found to be decreased when the expression of Pref-1 was high in the transgenic mice (Lee, Villena *et al.*, 2003). A recent study by Villena *et al.* (2008) showed that mice overexpressing Pref-1 are insulin resistant, even though they are protected from diet induced obesity (Villena, Choi *et al.*, 2008).

ADIPONECTIN

Adiponectin is another important gene regulating the adipocyte differentiation. Adiponectin appears to be mainly produced by adipocytes, and thus are characterized as an adipokine (Fantuzzi, 2005). Low circulating levels of adiponectin have been associated with obesity, insulin resistance, type 2 diabetes, and cardiovascular disease (Fu, Luo *et al.*, 2005). Adiponectin is exclusively expressed by mature adipocytes, and has both autocrine and paracrine effects in adipose tissue. The paracrine effect is illustrated by its role in adipocyte differentiation. Experiments have shown that adipocytes overexpressing adiponectin has a consistent accelerated adipocyte differentiation. Both increased lipid accumulation and insulin-responsive glucose transport activity in matured adipocytes are results from an accelerated adipocyte differentiation (Fu, Luo *et al.*, 2005; Lara-Castro, Fu *et al.*, 2007). Thus, adiponectin has a central role in the regulation of insulin sensitivity (Beltowski, 2003; Diez and Iglesias, 2003; Kahn, Hull *et al.*, 2006).

PLASMINOGEN ACTIVATOR INHIBITOR -1

Plasminogen activator inhibitor-1 (PAI-1) is part of the serine-protease inhibitors family and functions as an important inhibitor of fibrinolytic activity. Fibrinolysis is described as an enzymatic cascade reaction, where the result is degraded fibrin (Skurk and Hauner, 2004). Studies during the past years shows impaired fibrinolysis and increased levels of plasma PAI-1 in patients suffering from overweight or obesity (Giltay, Elbers *et al.*, 1998; McGill, Schneider *et al.*, 1994).

The role of PAI-1 in adipocytes is reported to promote the adipocyte differentiation in PAI-1 deficient mice. Some of the effects from PAI-1 deficiency are protection against a dedifferentiation by TNF- α and insulin resistance (Liang, Kanjanabuch *et al.*, 2006).

1.4 MACRONUTRIENTS LINKED TO OBESITY AND INFLAMMATION

Nutrients and metabolic excess are considered to be the triggering factors of inflammatory response in obesity (Bluher, 2008). It is believed that diet induced obesity in humans mainly is due to the amount of fat in the diet. However, the dietary fat intake in USA has decreased from 40 % of energy intake in the 1960s to around 33 % in 1995, while the dietary carbohydrate has increased from 45 % to 52 % of energy intake during the same period (Connor and Connor, 1997).

1.4.1 Dietary fat, n-6/n-3

Our ancestors consumed a larger amount of rich fruits, vegetables, lean meat and fish. The diet was less dense in calories but higher in fibers. As a result of this food intake, the diet was lower in total fat and saturated fat, but more important it contained an equal amount of both omega-6 (n-6) and omega-3 (n-3) (Simopoulos, 2000). N-6 and n-3 are designated essential fatty acids which the body can not synthesize itself, and must therefore be supplemented through the diet. The present study will mainly be focused on the dietary omega-6 (n-6) and omega-3 (n-3). In this thesis the omega nomenclature will be used, where n- signifies the position of the first double bond from the methyl end (figure 1.3).

OMEGA-3 (n-3)

Alpha-linolenic acid (ALA; 18:3n-3) is the major n-3 fatty acid. In the body, ALA is metabolized to the marine polyunsaturated fatty acids (PUFAs); eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). (Simopoulos, 2000). Seafood and oily fish including fish oil capsules are good sources of these fatty acids (Calder and Yaqoob, 2009). The daily intake of n-3 fatty acids is estimated to about 0.06 mg-2g in most Western countries (Zhou and Nilsson, 2001). The lower intake of n-3 PUFAs is partially due to the decrease of fish consumption along with industrial production of meat rich in n-6 fatty acids (Simopoulos, 2000).

OMEGA-6 (n-6)

Linoleic acid (LA; 18:2n-6) is the major n-6 fatty acid, and also the predominant PUFA in the Western diets (Simopoulos, 2000; Simopoulos, 2002b). The daily intake of LA is about 10-20g, which constitutes 85 % of total PUFA intake (Zhou and Nilsson, 2001). LA is metabolized to one of the eicosanoid precursors arachidonic acid (AA; 20:4n-6) in the body (Simopoulos, 2000). The increased consumption of n-6 during in the last 100 years is due to the development of technology and growing agribusiness and food processing. The mass production of vegetable oils after World War I became more efficient and not least more economical (Simopoulos, 2003). Some vegetable oils rich in n-6 fatty acids and very low in n-3 fatty acids are; corn-, safflower-, sunflower- and cottonseed oil. Corn oil and safflower oil have a n-6/n-3 ratio of 60:1 and 77:1 respectively (Simopoulos, 2001).

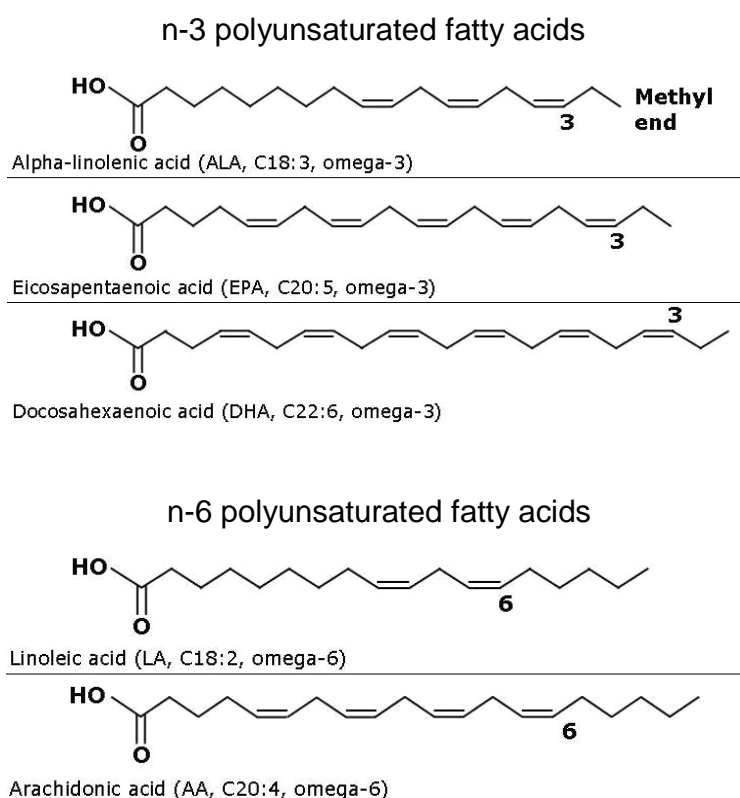


Figure 1.3: Overview of PUFA in the n-3 series and n-6 series. The omega nomenclature is used to signify the position of the first double bond from the methyl end.

From <http://www.eufic.org/article/en/nutrition/fats/artid/The-importance-of-omega-3-and-omega-6-fatty-acids/>

The imbalance of n-6- and n-3 fatty acids intake is reflected by the n-6/n-3 ratio, whereas a more physiological ratio is about 4:1, but the current ratio is about 20-30:1 in the Western diet (Sierra, Lara-Villoslada *et al.*, 2006). The increased n-6/n-3 ratio in the diet may contribute to increased incidences of inflammatory disorders including cardiovascular disease. We know today that n-3 fatty acids play an important role in both growth and development. It might also be a key factor in the prevention and treatment of inflammatory diseases and autoimmune disorders including cancer. (Simopoulos, 2002b)

1.4.2 Eicosanoids

Eicosanoids (from the Greek word eikosi for ‘twenty’) are signal molecules and part of the oxygenated metabolites family. Eicosanoids are involved in autoimmunity, allergic diseases and inflammation. The main classes of eicosanoids includes; prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT) (Harizi, Corcuff *et al.*, 2008). The 20-carbon PUFAs AA (20:4n-6) and EPA (20:5n-3) are the parent fatty acids for the production of eicosanoids (Simopoulos, 2003; Zhou and Nilsson, 2001). Whereas AA is the precursor of the common eicosanoids that act as mediators of inflammation, EPA, the n-3 counterpart to AA, is converted to eicosanoids that although structurally related, have different properties (Simopoulos, 2002a).

There is a partial replacement of n-6 fatty acids in the membrane in almost all cells when n-3 fatty acids are supplemented in the diet. AA and EPA compete for the same set of enzymes (blue text figure 1.4). The goal of EPA is prostaglandin and leukotriene synthesis of less inflammatory and chemotactic derivatives at the cyclooxygenase (COX) and lipoxygenase (LOX) level (Simopoulos, 2002b). AA results in an activation of pro-inflammatory mediators like PGE₂, LTB₄ and other related metabolites that can act as potent inflammatory mediators and producing cytokines (Bagga, Wang *et al.*, 2003). Conversely, the mediators produced by n-3 PUFAs; PGE₃ and LTB₅ have anti-inflammatory properties ((Calder, 2002; Gil, 2002) .

In terms of healthy and unhealthy adipose tissue, the type of dietary fat may be of particular importance. Obesity along with other chronic disorders such as cardiovascular disease, diabetes, rheumatoid arthritis are all associated with an increased production of the inflammatory components; TXA_2 , LTB_4 , $\text{IL-1}\beta$, IL-6 , $\text{TNF-}\alpha$, and C-reactive protein (CRP) (Simopoulos, 2003). All these factors increase by the increase in n-6 PUFAs intake, where n-3 PUFAs are reported to reduce the inflammatory response by displacing arachidonic acid in membrane phospholipids. Feeding rodents a diet high in n-3 PUFAs is reported to reduce plasma levels of cytokines such as $\text{TNF-}\alpha$, IL-6 and $\text{IL-1}\beta$ (Bhattacharya, Rahman *et al.*, 2007; Venkatraman and Chu, 1999), and adipose tissue inflammation induced by high-fat diet in db/db mice is prevented by fish oil (Todoric, Loffler *et al.*, 2006). The ingestion of fish oils in human can also lead to a decreased production of PGE_2 metabolites, followed by a decrease in TXA_2 and LTB_4 (Simopoulos, 2002b).

Thus, the n-6/n-3 ratio may be of importance and the increased consumption of n-6 PUFAs at the expense of n-3 PUFAs may aggravate the metabolic consequences of obesity. In this context it should also be noted that the increased consumption of farmed fish, which due to insufficient supplies of n-3 PUFAs contain a less favorable ratio of n-3 to n-6 PUFAs.

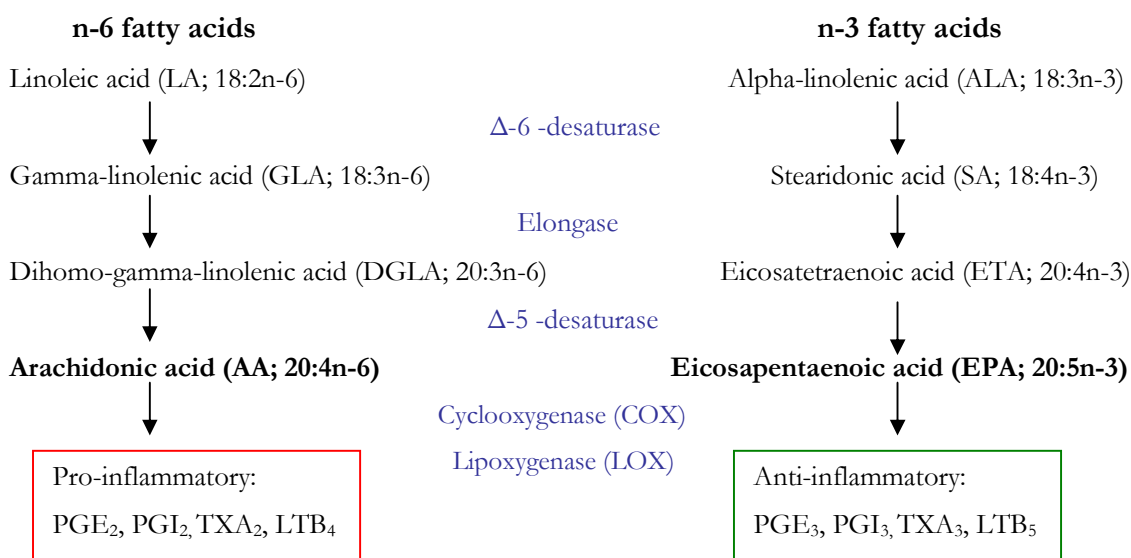


Figure 1.4: Synthesis of eicosanoids: prostaglandins (PG), leukotrienes (LT) and thromboxanes (TX) from n-3 and n-6 fatty acids. Modified from Ailhaud *et al.* (2006) and Wolters (2005).

1.4.3 Dietary carbohydrate

Carbohydrates can exist as monosaccharides, disaccharides or longer polymers of glucose. The dietary monosaccharides: glucose, fructose, and galactose are easily absorbed in the small intestine by enterocytes, and subsequently rapidly transported into the portal circulation. Disaccharides such as sucrose and lactose need to be cleaved into monosaccharides by the brush-border glucosidases in order to be absorbed (Flowers and Ntambi, 2009). Sucrose consists of one molecule of glucose and one molecule of fructose. Sucrose can be found in sugar cane, sugar beets, honey and corn syrup (Schaefer, Gleason *et al.*, 2009). It has been suggested that increased intake of sucrose in the diet that enters the bloodstream rapidly after ingestion, contribute to the increased prevalence of obesity and metabolic syndrome (Flowers and Ntambi, 2009).

There is a special connection between the macronutrients fat and carbohydrates because of the reflection in blood lipid concentrations. An increased level of sugar consumption gives a higher blood lipid concentration. (Chong, Fielding *et al.*, 2007). As mentioned earlier the carbohydrate intake has increased from 45 % to 52 % of energy intake during the years 1960s-1995 (Connor and Connor, 1997). It has been reported that a low-fat, high-carbohydrate diet is able to stimulate *de novo* lipogenesis in human. The concentration of plasma triglycerides was also shown to be proportionate increased in these subjects (Hudgins, 2000).

1.4.4 Dietary protein

Dietary proteins are broken down to peptides and amino acids in the digestive system. 20 amino acids function as building blocks for protein synthesis, where each amino acid has a basic structure of two carbon atoms and one nitrogen atom. In addition, a variable R-group gives each amino acid their unique character (Skålhegg, 2007).

The intake of dietary protein is recommended to be approximately 10-15 % of energy intake, when the individuals are in energy balance and has a stable weight (WHO, 2000). The average protein intake in the US was approximately 13.4% in children (4-8 years) to 16.0% in men (51-70 years) between 2003-2004 (Fulgoni, 2008). Protein has a high satiating effect,

which makes it central in the achievement of body weight loss and body weight maintenance (Astrup, Meinert Larsen *et al.*, 2004). A high protein intake also leads to an increased energy expenditure, which might be due to the gluconeogenesis and urea synthesis (Westerterp-Plantenga, Nieuwenhuizen *et al.*, 2009).

A decrease in energy intake has been reported in rodents fed a high-protein, low-carbohydrate diet. The results were also a lower body weight among these animals due to a reduced adipose tissue mass and less development of enlarged adipocytes (Blouet, Mariotti *et al.*, 2006). There has been suggested that a high-protein diet ingested over time could protect against metabolic syndrome (Lacroix, Gaudichon *et al.*, 2004). However, the mechanism which link dietary protein and obesity development is poorly understood.

1.5 AIM OF THE STUDY

The theory presented in this introduction chapter has been aimed to give an overview of how obesity can be linked to insulin resistance and inflammation. Our basic hypothesis is that dietary-induced inflammation in adipose tissue can be modulated by the type of dietary fatty acids, the n-3/n-6 ratio in particular. We assume that increasing the ratio of n-3 fat in the diet attenuates inflammation. Earlier studies in this laboratory have demonstrated that the obesigenic effect of corn oil is determined by the carbohydrate to protein ratio in the diet (Madsen, Pedersen *et al.*, 2008). Thus, we predict that the carbohydrate to protein ratio also may influence the inflammatory response.

The primary aim for this study is:

To find out whether the type of dietary fat can modulate inflammation

Furthermore, we wanted to base the study on these following questions:

- What are the effects of the different diets on body weight- and obesity development?
- What are the effects of the different diets on adipose tissue inflammation?
- What are the effects of the different diets on metabolism?



2 MATERIALS AND METHODS

2.1 ANIMAL MODELS

Thirty five male C57BL/6J mice, approximately 11 weeks of age were obtained from Taconic Europe, (Ejby, Denmark). The animals' weight ranged from 23.35 to 33.44g, with a mean weight of 27.66 ± 2.59 g at time of receipt. All animal protocols used in the experiment were approved by the Norwegian State board of biological experiments.



Figure 2.1: C57BL/6J mouse. From http://www.taconic.com/user-assets/Images/Products-Services/em_mod_black.jpg (Taconic Farms Inc, 2006-2009).

2.2 EXPERIMENTAL SET-UP

During the acclimatisation period, the mice were fed a low energy diet (chow) and tap water *ad libitum*. After one week of acclimatisation, the mice were divided into 5 groups (A, B, C, D and E) with 6 animals in each group. The extra 5 mice were housed together in a separate cage. The division of the groups were based on random selection of the mice, where the mouse's start weight was registered. Small adjustments were made within the groups to achieve approximately the same mean weight in each group. The mice were housed in individual cages, and kept in a 12h light/dark cycle at 22-23 °C and approximately 50 % humidity, during the entire 10 weeks feeding experiment. The cages with wooden chip bedding (Scanbur Bedding Aspen, Norway) were cleaned and changed every second week. Also during the experiment the mice had free access to food and tap water. The amount of food given was weighed and registered on Monday, Wednesday and Friday mornings (10 a.m.). The remaining food in each cage were also weighed and registered twice a week. The mice's body weights were recorded every Monday morning (10 a.m.).

2.3 DIETS

The diets were obtained from Ssniff (Spezialdiäten GmbH, Soest, Germany), and stored at -20 °C in small buckets with lids.

The diets given were; low energy to the control group A , high-fish oil, high-protein (fish oil + protein) to group B, high-fish oil, high-sucrose (fish oil + sucrose) to group C, high-corn oil, high-protein (corn oil + protein) to group D, and high-corn oil, high-sucrose (corn oil + sucrose) to group E. The amount of ingredients added in the different diets including their respective treatment groups are listed in table 2.1.

To verify the enrichment of n-3 and n-6 PUFA in the fish oil and corn oil enriched diets, respectively, fatty acid composition was measured (see appendix I).

Table 2.1: Amount of ingredients in the different diets given in g/kg diet.

Group	(g/kg diet)				
	A	B	C	D	E
	Low energy/ Control Diets # 110750	High-fish oil- high protein	High-fish oil- high sucrose	High-corn oil- high protein	High-corn oil- high sucrose
Ingredient					
Casein	200	540	200	540	200
L-Cystine	3	3	3	3	3
Starch (normal)	529,486	9,486	9,486	9,486	9,486
Sucrose	90	90	430	90	430
Cellulose	50	50	50	50	50
Corn oil	70	70	70	250	250
Fish oil	-	180	180	-	-
t-Butylhydroquinone	0,014	0,014	0,014	0,014	0,014
Salt mix (Dyets #210025)	35	35	35	35	35
Vit mix (Dyets #310025)	10	10	10	10	10
Choline, Bitartrate	2,5	2,5	2,5	2,5	2,5
Supplement (Diets # 410750)	10	10	10	10	10
Total	1000	1000	1000	1000	1000

2.4 BODY WEIGHT AND FEED INTAKE RECORDING

The mice's bodyweight and feed intake pattern can be used as a good indicator for the mice's contentment and development. The weights were recorded every Monday morning until the day they were sacrificed, 21 weeks old. The feed intake was calculated from weighing the amount of food given 3 times (Monday, Wednesday and Friday) a week and the remaining food in the cage.

2.5 DATA COLLECTION

After 10 weeks of feeding, the mice were terminated. They were also fed the day before, because of lipogenic activity measurements later in the experiment.

The mice were weighed before being anaesthetized with Isofluran (Isoba-vet., Schering-Plough, Denmark), using the apparatus Univentor 400 Anaesthesia Unit (Univentor Limited, Sweden), and killed by cardiac puncture.

2.5.1 *Blood samples*

The sternum was cut open, and a syringe was placed carefully in the heart to collect the blood sample in small tubes containing EDTA as anticoagulant. The samples were immediately centrifuged at 2500 x g and 4 °C for 5 minutes, to separate plasma and red blood cells (RBC). The samples were then stored at -80 °C until subsequent analysis.

2.5.2 *Adipose-, liver- and muscle tissue*

Fat deposits: Two visceral white adipose tissue depots; epididymal (eWAT) and retroperitoneal (rWAT), one subcutaneous white adipose tissue depot; inguinal (iWAT) (figure 2.2), and one brown adipose tissue depot; intrascapular (iBAT) (figure 2.3) were excised and weighed. The tibialis anterior muscle and the liver tissue were also removed from the animal and weighed. The tissues were quickly frozen in liquid nitrogen, and stored at -80 °C until analyzed. Additionally, photographs of one representative mouse within each group were also taken during the dissection. (Presented in section 3.1.2, figure 3.7.)

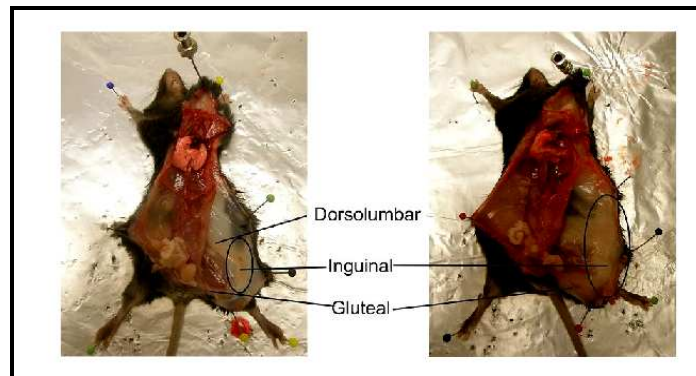


Figure 2.2: Posterior subcutaneous adipose tissue in C57BL mice. The inguinal white adipose tissue (iWAT) is circled out.



Figure 2.3: Interscapular brown adipose tissue (iBAT) in lean (left) and obese (right) C57BL mice.

2.5.3 Histology

Tissue samples of eWAT, rWAT, iWAT, iBAT liver and muscle (t. anterior) from two mice that were best representative from each group, based on body weight development, from each group were fixed in 4 % formaldehyde. Only eWAT and iWAT were further assessed in histology methods in the present study.

2.6 CHEMICALS AND REAGENTS

All chemicals and reagents were obtained from commercial sources. See appendix II, III, IV, V, VI and VII for details.

2.7 ANALYTICAL METHODS

Total RNA was isolated with Trizol from the eWAT, iWAT, rWAT, iBAT, liver tissue and tibiales anterior from individual mice. In order to assess the purity and quantity of isolated RNA, the samples were measured using the Nanodrop (Saveen Werner, Sweden). Measurements of integrity on the Bioanalyzer (Agilent Technologies, USA) were made to make sure that the isolated RNA was intact. The isolated RNA could then be transcribed to cDNA in the RT-reaction, and then finally quantitative real-time PCR could be performed, for the assessments of gene expression levels.

More detailed information about chemical and reagents is listed in appendix (II, III, IV and V). All analytical methods were performed at laboratory for molecular biology (NIFES).

2.7.1 RNA extraction with Trizol

Principle

Tissue is homogenized in Trizol, which contains among other things, phenol and guanidinium salts. RNA is separated from proteins and deoxyribonucleic acid (DNA), when adding the chloroform. RNA is present in the water phase, while proteins are in the organic and interphase, and DNA is in the interphase. RNA is then extracted from the water phase by adding isopropanol. The DNA free kit, containing the enzyme DNase is used to eliminate any remaining DNA (NIFES, 2005b).

Procedure

1 ml of Trizol reagent was added to each tube with tissue samples and homogenized with a homogenizing instrument (Precellys 24 lysis & homogenization instrument, Bertin Technologies), using zirconium beads. The samples were centrifuged at 12000 x g for 10 minutes at 4 °C (Eppendorf Centrifuge 5415 R), and incubated for 5 minutes in room temperature. 200µl chloroform was added to each sample and shaken well for 15 seconds. The samples were then again incubated for 2-3 minutes in room temperature. After incubation the samples were centrifuged at 12000 x g for 15 minutes at 4 °C. The centrifugation separated the samples into a phenol-chloroform phase, an interphase and a colorless upper aqueous phase. The top layer – aqueous phase from all samples was transferred to new sets of eppendorf tubes. 500 µl of isopropanol was added to each tube

and mixed well. The tubes were then incubated for 10 minutes at room temperature followed by 10 minutes in the refrigerator (4 °C). After incubation the samples were centrifuged at 12000 x g for 10 minutes at 4 °C. The supernatant was discarded with a vacuum apparatus (IBS Integra Biosciences, Vacuboy, Switzerland). The RNA pellet in each tube was washed with 1000µl 75% ethanol, and vortexed. The samples were centrifuged again at 75000 x g for 5 minutes at 4 °C. The supernatant was discarded again as previous and incubated for 2-3 minutes on ice. 50-300 µl of MilliQ water was added to the tubes to dissolve the pellet. The RNA samples were further purified using the DNA free kit, according to the vendor's description to eliminate any remaining DNA.

2.7.2 RNA quality and quantity on the Nanodrop ND-1000

Principle

The Nanodrop ND-1000 spectrophotometer (Saveen Werner, Sweden) enables highly accurate analyses of small samples. Surface tension is used to hold a column of liquid sample in place while a measurement is made, see figure 2.4c). Measurements at 260 nm and 280 nm give an indication of the quality or purity of the sample. At 280nm of DNA, protein and phenol are measured (NIFES, 2005b).

Purity

It is preferable to have a A260/A280 ratio $\geq 1.7 - 2.1$. A ratio lower than 1.6 might indicate that RNA has not completely dissolved in the water or protein remnants. The protein remnants will possibly degrade RNA under storage. A ratio higher than 2.2, might indicate phenol remnants in the sample (NIFES, 2005b). Measurements at 280 nm do not provide information about possible residual organic contaminations considered at 230 nm. Thus, it is preferable to have a A260/A230 ratio > 1.8 (Imbeaud, Graudens *et al.*, 2005).

Concentration

It is preferable to have a RNA concentration between 100-3000 ng/µl. The RNA concentration is important when performing quantitative real-time PCR. (Dilution are made down to 25ng/µl, thus samples must contain enough RNA to run qPCR.)

Procedure

The quality of RNA was assessed by measuring the absorbance A260 and A280 with the Nanodrop ND-1000 spectrophotometer (Saveen Werner, Sweden). This was performed by placing only 2 μ l of sample directly on to one measurement pedestal, see figure 2.4 b). The absorbance ratio and concentration was shown on the screen, after approximately 10 seconds (Ayubi, 2007b). When the measurements were complete, the sample was wiped from both the sample arm and sample pedestal using a soft tissue, see figure 2.4 d).

Samples with a lower A260/230 ratio than 1.7 were precipitated with 3M NaAc pH 5.2 and EtOH and incubated at -80 °C over night. The samples were then centrifuged (Eppendorf Centrifuge 5415 R) at 12000 x g for 10 minutes. The supernatant was discarded with vacuum apparatus (IBS Integra Biosciences, Switzerland) before measurements on nanodrop again the following day.

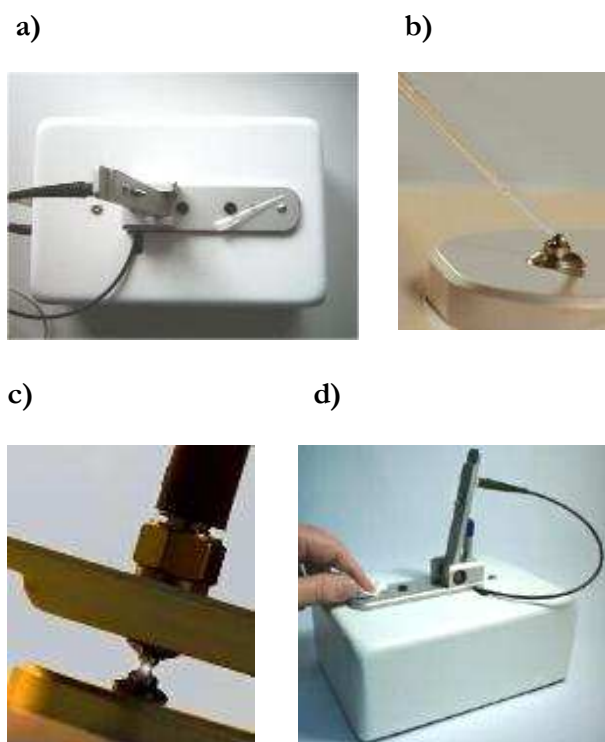


Figure 2.4: Nanodrop ND-1000 Spectrophotometer. a) An open sample apparatus. b) A droplet of sample is pipetted on to the measurement pedestal. c) Surface tension holds the sample in place. d) After measurements, the sample is wiped from the measurement pedestals with a soft tissue. Figure adapted and modified from Ayubi (2007).

2.7.3 RNA integrity on the Bioanalyzer (RNA 6000 Nano)

Principle

The RNA LabChip consists of micro-channels and an interconnected network of fluid reservoirs. The chips are fabricated from glass and contain 16 wells: 12 for nucleic samples, 3 for loading the gel-dye mixture, and 1 for a molecular size ladder (Panaro, Yuen *et al.*, 2000). The chip becomes an integrated electrical circuit once the wells and channels are filled. Small amounts of RNA samples are separated in the channels of the LabChip according to their molecular weight. Charged RNA is driven by a voltage gradient. Because of a constant mass-to-charge ratio and the effect of a sieving polymer matrix, the molecules are separated by size (Ayubi, 2007a). The results are displayed as a gel-like image and peaks on an electropherogram that provides a detailed visual assessment of the RNA quality (see figure A.1 in appendix IX) (Mueller, Lightfoot *et al.*, 2004). The RNA 6000 ladder is used as both a sizing and quantification standard (Sodowich, Fadl *et al.*, 2007).

RIN (RNA Integrity Number)

The RNA 6000 Nano LabChip Kit was used to evaluate the RNA integrity. The RNA integrity number (RIN) was defined for the classification of total RNA, based on a numbering system from 1 to 10, with 1 being totally degraded RNA and 10 being fully intact RNA (Schroeder, Mueller *et al.*, 2006). Figure 2.5 shows electropherograms of samples with varying levels of intactness and respectively RINs. A RIN software algorithm was used to classify the samples (Mueller, Lightfoot *et al.*, 2004).

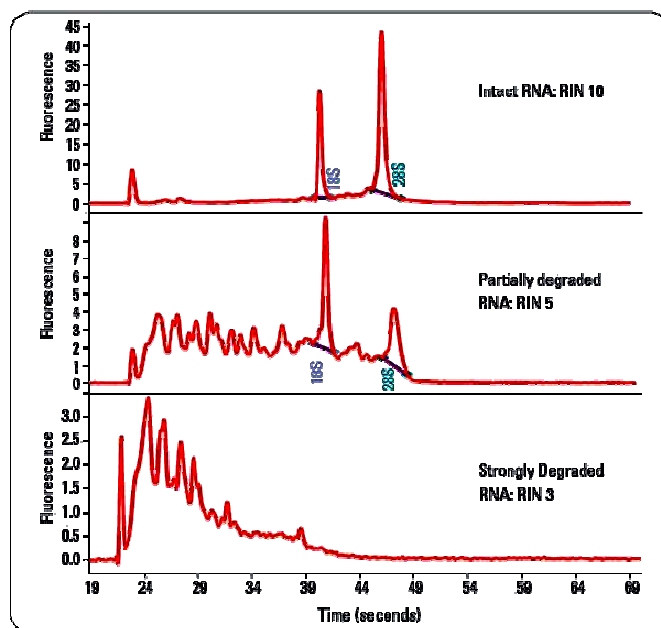


Figure 2.5: Electropherograms of 3 different samples of varying intactness, from the top: high- quality intact RNA with two distinct ribosomal peaks which corresponds to either 18S or 28S for eukaryotic samples and RIN 10. The electropherogram in the middle shows a partially degraded sample, with RIN 5. On the bottom is a strongly degraded RNA sample with RIN 3. Modified from Mueller, Lightfoot et al. (2004).

Procedure

A selection of RNA samples were analysed on the Bioanalyzer. A more detailed description of the procedure is to be found in vendor's protocol (Agilent Technologies, 2003). (See appendix III for more detailed information about reagents)

The RNA 6000 gel matrix was centrifuged at 1500 x g for 10 minutes (Eppendorf Centrifuge 5415 R). 32.5 μ l filtered gel was aliquoted into 0.5 ml RNase free tubes. The RNA 6000 Nano dye concentrate was then vortexed for 10 seconds, and spun down. 0.5 μ l of dye was added to the tube with 32.5 μ l filtered gel. The solution was vortexed well and centrifuged at 13000 x g for 10 minutes. 9 μ l of gel-dye was added to RNA chip on the Chip Priming Station. 5 μ l of RNA 6000 Nano marker was added to the well marked with a ladder symbol. 1 μ l of RNA 6000 ladder was pipetted into the well marked with the ladder symbol. 1 μ l of RNA sample was pipetted in each of the 12 sample wells. The chip was run on Agilent 2100 Bioanalyzer (Agilent technologies, USA) within 5 minutes.

2.7.4 RT-reaction (Reverse Transcription)

Principle

In the RT-reaction, RNA is reverse transcribed to complementary DNA (cDNA) by using the enzyme reverse transcriptase. Generated cDNA can be used to determine the changes in gene expression levels in the quantitative real-time PCR (Valasek and Repa, 2005).

Procedure

cDNA was synthesized from total RNA of each sample from 5 tissues (eWAT, iWAT, rWAT, iBAT and liver), by using Multiscribe reverse transcriptase (50 U/ μ l) with oligo d(T)₁₆ primer (50 μ M) in a 30 μ l reaction. The RNA concentration was measured with nanodrop as previous (RNA quality on Nanodrop ND-1000). RNA or ddH₂O was added to achieve the desired concentration of 30 ± 1 ng/ μ l. The final concentration and volume of the different reagents in the RT- reaction mix is listed in table 2.2. The volume of each reagent was multiplied by 100 (96 wells + dead volume).

Table 2.2: RT -reaction mix for a 30 μ l reaction

Reagent	Volume (μ l)	Final concentration
RNase free ddH ₂ O	1.3	
TaqMan RT buffer 10X	3.0	1 X
25 mM Magnesium chlorid	6.6	5.5 mM
10 mM deoxyNTPs	6.0	500 μ M per dNTP
50 μ M Oligo d(T) ₁₆ primer	1.5	2.5 μ M
RNase inhibitor (20 U/ μ l)	0.6	0.4 U/ μ l
Multiscribe reverse transcriptase (50 U/ μ l)	1.0	1.67 U/ μ l

The RT-reactions were run on 96-well reactions plates (AB gene PCR plate, Thermo Scientific) in duplicate. 20 μ l of reaction mix and 10 μ l RNA were pipetted in each well on the 96-well RT plate. Two negative controls: non amplification control (nac) and non template control (ntc) were also run for quality assessment. The nac is lacking the multiscribe transcriptase enzyme, and the ntc control has no RNA template. A 96- well full plate cover

was put on the RT plate as a lid before centrifugation at 50 x g for 1 minute (Eppendorf Centrifuge 5810 R). The 96-well RT plate was the run on Gene Amp PCR System 9700 PCR machine (Applied Biosystems). The thermal cycling parameters for RT reactions are shown below in table 2.3.

Table 2.3: Reverse Transcriptase reaction conditions, taken from "279-RT Reaction" method description with modifications (NIFES, 2005a)

Step	Incubation	RT	Reverse Transcriptase Inactivation	End
	HOLD	HOLD	HOLD	HOLD
Temperature (°C)	25	48	95	4
Time (minutes)	10	60	5	∞

The synthesized cDNA was diluted 2 X with MilliQ water into a 60 µl solution. The RT - plates were then stored at -20 °C until use.

2.7.5 Determination of gene expression levels by Quantitative Real-Time PCR

Quantitative real-time polymerase chain reaction (qPCR) was applied to measure the gene expression levels from the eWAT, iWAT, rWAT, iBAT and liver. β -actin, TATA box binding protein (TBP) and calnexin were used as housekeeping genes based on their high stability (Ajuwon and Spurlock, 2005b; Tatsumi, Ohashi *et al.*, 2008; van Schothorst, Keijer *et al.*, 2006). The list of housekeeping genes and target genes, with their sequences used in qPCR is shown in appendix V, table A.6.

Principle

qPCR is a method based on amplification of short DNA base sequences within a longer double stranded DNA molecule. In this case, the target DNA sequences are synthesized cDNA from RT-reaction. By using sequence-specific primers and a DNA-binding dye in the PCR reaction, it is possible to monitor fluorescent signal proportional to the amount of product formed during an amplification cycle (Valasek and Repa, 2005). SYBR GREEN is a fluorogenic- and double stranded DNA (dsDNA) binding dye, which binds to all dsDNA in a PCR reaction. SYBR GREEN exhibits little fluorescence in unbound state, but upon

excitation emits a strong fluorescent signal (figure 2.6) (Ma, Shieh *et al.*, 2006; Smith and Osborn, 2009). Thus, the greater the amount of dsDNA present, the greater the amount of fluorescent signal from SYBR GREEN (Valasek and Repa, 2005).

There are three major steps in a PCR reaction:

1. **Denaturation at 95 °C:** High temperature denatures the DNA strands and melts dsDNA into single strands (Valasek and Repa, 2005).
2. **Annealing at 60 °C:** The temperature is lowered and primer binds to their specific sites (Ma, Shieh *et al.*, 2006).
3. **Extension at 72 °C:** Extension of a complementary strand from each annealed primer (Smith and Osborn, 2009).

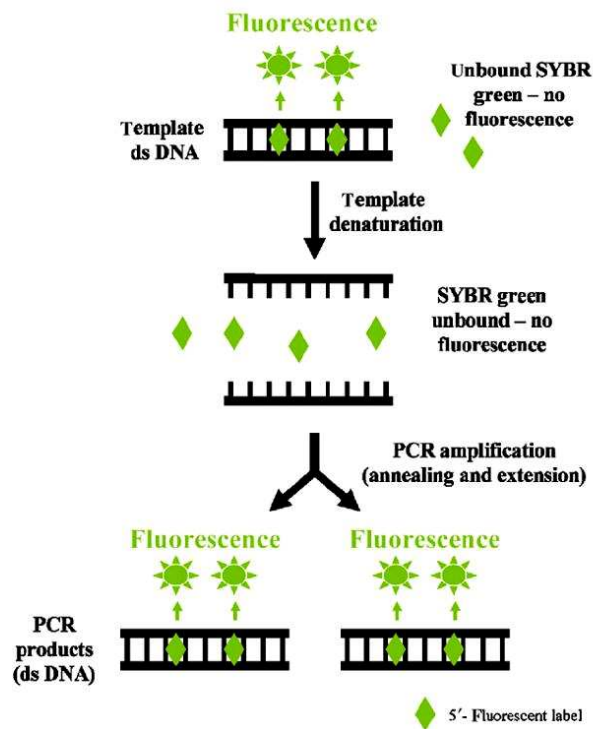


Figure 2.6: SYBR GREEN has the ability to bind to all double-stranded DNA qPCR products, and emit a fluorescent signal. Modified from Smith and Osborn (2009).

Procedure

Reaction mix was made with 500 μl SYBR GREEN Master reagent, 5 μl of primer pair forward and reverse (100 μM each primer) and 290 μl MilliQ water (see appendix for details in table A.5). The volume of the reagents listed in table 2.4 was multiplied by 100 (number of Real time reactions (96) + dead volume) when making the reaction mix. 18 μl of reaction mix and 2 μl of cDNA (30 ng/ μl) from the RT-reaction were then transferred to a 384-well reaction plate, using Biomek 3000 Laboratory Automation Workstation (Beckman Coulter, USA). qPCR was performed on Light Cycler 480 Real- Time PCR System (Roche, Norway), with SYBR GREEN Master reagent in a 20 μl reaction.

Table 2.4: SYBR GREEN reaction mixture of 20 μl reaction.

Reagent	Volume (μl) per sample
ddH ₂ O	2.9
Primer forward (100 μM)	0.05
Primer reverse (100 μM)	0.05
SYBR GREEN Master	5

Thermal cycling conditions for the qPCR reactions consist of an initial denaturation step at 95 °C for 5 minutes, followed by 45 cycles of 10 seconds denaturation at 95 °C, 20 seconds annealing at 60 °C, and 30 seconds extension at 72 °C. All samples were run in duplicate, while the standard was run in triplicate. The software programme graphs the amplification curves with cycle threshold (C_T) values shown on the screen. The C_T value is inversely proportional to the amount of specific nucleic acid in the sample, and shows when the fluorescence reaches a threshold level (Valasek and Repa, 2005). The software also calculates the sample's efficiency and error. An efficiency value between 1.8 -2.2 and an error value less than 0.04 were considered as acceptable in this study.

2.7.6 Fatty acid composition by GLC

The method for determination of fatty acid composition by Gas Liquid Chromatography (GLC) of adipose tissue (eWAT, iWAT rWAT, iBAT), liver tissue, and red blood cells (RBC) were performed by Else Leirnes at the nutrient laboratory (NIFES).

Principle

- Extraction of fat from sample: Total lipid content from the samples was extracted using chloroform-methanol 2:1.
- Filtration: The extract was filtrated to remove tissue and protein remnants
- Evaporation: Chloroform-metanol was evaporated.
- Saponification and methylation
- Analytical GLC
- Determination with flame ionisation detector
- Calculation of % distribution and amount (mg/g) of the different fatty acids (NIFES, 2009)

2.8 HISTOLOGICAL METHODS

In order to evaluate the obesity development and inflammation state in the different mice adipose tissues with microscopy, slides with sections were made after fixation, dehydration and embedding of the tissues. The sections were then subjected to standard hematoxylin and eosin staining (H&E). Cell size of eWAT and iWAT from the different groups was compared by using the microscope.

For a more detailed description of chemicals and reagents see appendix VI. All histology methods were performed at laboratory for microbiology (NIFES).

2.8.1 Fixation and dehydration

Fixation is used to preserve the tissues' microscopic structure and morphology and to prevent tissue digestion by enzymes or bacteria. Fixation can also enhance tissue staining (Cormack, 2001; Uchôa Junqueira and Carneiro, 2005).

The tissues were fixed in 4% formaldehyde after tissue collection (see appendix table A.7 for chemicals and reagents). A 0.1 M phosphate buffer (PB) with pH 7.4 was made by mixing $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$ (0.027 mol) and $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ (0,077 mol) and solved with ddH₂O using a magnet stir. The PB was diluted with 1000 ml ddH₂O and pH adjusted. The tissues in formaldehyde were washed with PB twice using small plastic pipettes.

Manuel dehydration was carried out in order to eliminate the fixative and water from the tissue, and replace it with dehydration solution. The PB was replaced with 50% EtOH and put on a mixing machine (HS 501 D, JANKE & KUNKEL, IKA ® Labortechnik, Germany) for 1 hour. The 50 % EtOH was replaced with new solution of 50 % EtOH and mixed on the machine for 1 hour. This was repeated 4 times. Finally 50% EtOH was replaced with 70% EtOH and placed in the refrigerator at 4 °C until further treatment.

2.8.2 Dehydration on STP 120 and clearing with xylene

Before embedding the tissues with paraffin, further dehydration of water from the tissues were performed since paraffin is not soluble in water. Microflow II cassettes (McCormick Scientific, USA) with tissue from eWAT and iWAT were placed in a basket and dehydrated on the Spin Tissue Processor, (STP 120, Microm International GmbH, Germany). The tissues were placed in different baths starting from 70% to 100% ethanol. The program chosen is listed below in table 2.5. As a step in the dehydration process, ethanol is replaced with xylene for clearing of the tissues. Xylene is a paraffin solvent miscible with alcohol (Cormack, 2001).

Table 2.5: Dehydration program on STP 120

Dehydration program of adipose tissue from mice		
Bath	Reagent	Time
1	70 % EtOH	1 hour
2	80 % EtOH	1 hour
3	96 % EtOH	1 hour
4	96 % EtOH	1 hour
5	100 % EtOH	1 hour
6	100 % EtOH	1 hour
7	Xylene	10 minutes
8	Xylene	10 minutes
9	Paraffin	3 hours
10	Paraffin	2 hours

2.8.3 Paraffin embedding and sectioning

Embedding of the tissues was performed to firm them and make it possible to obtain thin sections. The tissues were embedded in paraffin (Histowax, Histolab products AB, Sweden) using EC 350 Paraffin embedding center (Microm International GmbH, Germany). The cassettes with tissues were put in liquid paraffin (59 °C), and subsequently placed on a metal shapes with paraffin and the cassette (without lid) on top. The embedded metal shapes with tissues were then transferred to the cooling plate, and taken out of the shapes after approximately 10 minutes.

A microtome (Leica RM2165, Germany) was used to cut 8µm thin sections of the embedded tissues. A bowl with water and a couple of drops 50 % methanol was put on a slide warmer (Slide warmer SW85) at approximately 60 °C (water temperature at 45 °C). The thin sections were carefully removed from the microtome and placed upon the water surface with a small brush. The methanol in the water bath was added to help the sections stretch better. The sections were transferred to glass slides marked with a pencil, and left dry over night in room temperature before staining.

2.8.4 Hematoxylin and eosin (H&E) staining and mounting

In order to examine the slides with a microscope, the sections must be stained. Hemtatoxylin and eosin (H&E) is the most commonly used combination of dyes. Hematoxylin stains the cell nucleus blue, while eosin stains the cytoplasm pink (Uchôa Junqueira and Carneiro, 2005).

All sections were stained with hematoxylin and eosin as followed by the procedure listed in table 2.6. The sections were then mounted with xylene based mounting medium (Microscopy Entellan, Merck, Germany).

Table 2.6: Hematoxylin and eosin dying procedure.

Staining paraffin embedded sections with hematoxylin and eosin	
Reagent	Time
Xylene	5 minutes
Xylene	5 minutes
100 % EtOH	5 minutes
90 % EtOH	5 minutes
80 % EtOH	5 minutes
70 % EtOH	5 minutes
50 % EtOH	5 minutes
dd H ₂ O	5 minutes
Hematoxylin	3 minutes
H ₂ O (warm tap water 40-45 °C)	5 minutes
dd H ₂ O	Rinse
Eosin	30 seconds
90 % EtOH	Wash
90 % EtOH	5 minutes
100 % EtOH	5 minutes
Xylene	5 minutes
Xylene	5 minutes

2.8.5 Microscopy pictures

The slides with sections of iWAT and eWAT of one mouse from each treatment group (A, B, C, D and E) were visually examined by using an Olympus BX 51 binocular microscope (System microscope, Japan), fitted with an Olympus DP50 3.0 camera. The slides were viewed at 400X magnification, scale bar 50 µm. After the overall assessment of the section, a representative field from iWAT and eWAT of each treatment group was photographed using the software program Cell[^]B V. 2.6.

2.9 PLASMA ANALYSIS

2.9.1 Determination of plasma parameters on the MAXMAT™ PL

80 µl plasma from each sample (n=28) was used to analyse the following parameters listed in table 2.7 on an autoanalyzer (MAXMAT SA, France) by Leikny Fjeldstad.

The MAXMAT™ PL work as a fully automated multi-purpose analyzer where micro plates are used for its reaction support (MAXMAT PL, 2003).

Table 2.7: Plasma parameters measured on MAXMAT PL.

Plasma analysis on MAXMAT PL
Glucose (glc)
Triacylglycerol (TAG)
D3-hydroxybutyrate (8-OH-but)
Non-esterified fatty acids (NEFA-C)
Alanine aminotransferase (ALT)
Lactate dehydrogenase (LDH)
Glycerol

2.9.2 Determination of insulin in plasma by ELISA

Insulin Mouse Ultrasensitive ELISA kit (DRG Instruments gmbH, Germany) was used for quantitative determination of insulin in plasma according to the vendor's instructions. For reagents see appendix VII, table A.8.

Principle

The method is based on a direct sandwich technique where two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. Incubation of the sample enables insulin to react with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies which are bound to the microtitration well. The washing step removes unbound enzyme labeled antibody, and the bound conjugate is detected by reaction with 3,3',5,5'- tetramethylbenzidine. Acid is added to stop the reaction and the colorimetric endpoint is read spectrophotometrically (DRG Diagnostics, 2007).

Procedure

Each determination was performed in duplicate for standards and samples. The reagents and samples were brought to room temperature before use. 25 μ l of standard 0 was added to each well on the 96-well plate before addition of samples and standards. 5 μ l of standards 0, 3-7 were added to each standard well, and a subsequent adding of 5 μ l sample in each sample well. 50 μ l of enzyme conjugate was added to both wells with standards and samples. The plate was incubated on a shaker (700-900 rpm) for 2 hours at room temperature. After incubation the plate was washed 6 times with an automatic plate washer (Skan Washer 300 Version B, Skatron Instruments, USA) and tapped firmly against absorbent paper. 200 μ l of TMB solution was added to each well, followed by incubation for 30 minutes. 50 μ l of stop solution was then added to each well. To ensure well mixing of substrate solution and stop solution, the plate was shaken before absorbance measurements at 450 nm and 620 with a spectrophotometric plate reader (iEMS Reader MF, Labsystems, Helsinki).

2.10 STATISTICAL ANALYSIS

2.10.1 *Microsoft Excel*

Microsoft® Excel 2002 SP3 was used to calculate the mean and standard error of the mean (SEM) of the data. All the results are presented as means \pm SEM.

2.10.2 *geNorm*

geNorm is a statistical algorithm which has been developed to evaluate the stability (M) and suitability of housekeeping genes. The genes with the lowest M values are considered to have the most stable expression across time. The geNorm program is based on the principle that the expression ratio of two ideal internal control genes is not changed under the experimental conditions (Tatsumi, Ohashi *et al.*, 2008).

β -actin, TBP and calnexin were used as housekeeping genes in the study, and the geNorm (v. 3.5) VBA applet for Microsoft Excel was used to determine the most stable gene (Vandesompele, De Preter *et al.*, 2002). $M < 1.5$ was considered as stable, however the normalisation factor was used to normalise by in this study. Gene expression data was calculated from C_T values obtained from qPCR, using the geNorm program. Quantity values were divided with normalisation factor to achieve the values of gene expression level.

2.10.3 *Statistica*

Data was statistical analysed using Statistica 8.0 (Statsoft Inc. USA, 2008). The treatment groups regarding body weight gain, feed intake, organ weights, fatty acid composition, gene expression- and plasma levels were statistical analysed as followed:

- Levene's test was used to test for homogeneity of variance.
- Kruskal-Wallis, a nonparametric test was performed to compare the groups when the Levene's test showed significant difference.
- Shapiro Wilk's W test was used to test for normality.
- One-way analysis of variance (ANOVA) was used when there was no significant difference in variance and normality, followed by post-hoc Tukey's HSD test to detect which groups were significant different from each other.
- P values < 0.05 were considered as significant different.

3 RESULTS

3.1 THE EFFECT OF THE DIFFERENT DIETS ON BODYWEIGHT- AND OBESITY DEVELOPMENT

To test the study's basic hypothesis that inflammation in adipose tissue can be modulated by the type of dietary fatty acids, the n-3/n-6 ratio in particular, mice were fed a high fat diet enriched in fish- or corn oil for 10 weeks. Since it was assumed that other dietary factors, such as the carbohydrate to protein ratio may influence the response, the high fat diets were enriched with either sucrose or protein. The control mice received a low energy diet. During the feeding experiment, one mouse from group B (fish oil + protein) and group C (fish oil + sucrose) were taken out, because of irregular weight gain and abnormal behaviour. The remaining 28 mice continued with the feeding experiment.

3.1.1 Body weight development

The body weight development and the organ weights of the different treatment groups were initially assessed to monitor the development of obesity. As expected, according to earlier studies (Madsen, Pedersen *et al.*, 2008), the mice receiving the high corn oil diet gained more weight when fed corn oil in combination with sucrose than protein (figure 3.1). However, rather surprising, also fish oil appeared to be significantly obesigenic when given in combination with sucrose.

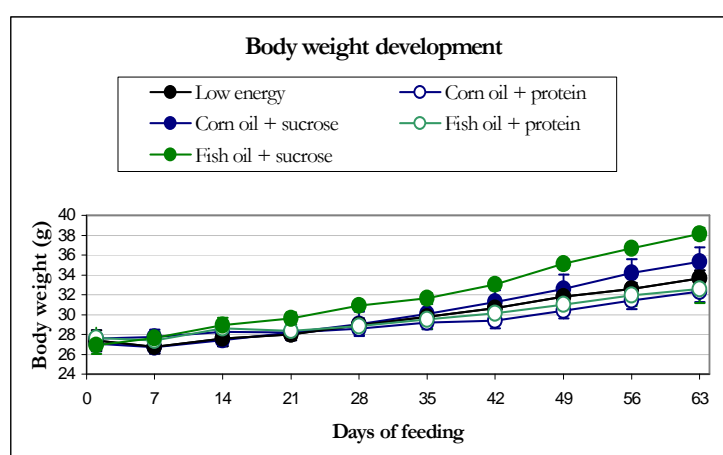


Figure 3.1: Body weight development of the C57BL/6J mice. The curves show the mean weight of the animals' from each of the 5 treatment groups during the feeding experiment of 63 days.

3.1.2 Adipose tissue and liver weights

One representative mouse from each treatment group was photographed during the dissection to determine the level of obesity. Adipose tissue and livers were dissected and weighed. Comparison of absolute organ weights indicate that the fat deposits from eWAT, iWAT, rWAT and iBAT has responded to the sucrose given in the diets.

eWAT: The weight of eWAT from the corn oil + sucrose group was significantly higher than the low energy (control) group ($P < 0.004$), the fish oil + protein group ($P < 0.0002$) and the corn oil + protein group ($P < 0.0003$) (figure 3.2). eWAT weights from the fish oil + sucrose group was also significantly higher than for the treatment groups receiving protein; fish oil + protein ($P < 0.002$) and corn oil + protein ($P < 0.003$), but not significantly different compared to the control group.

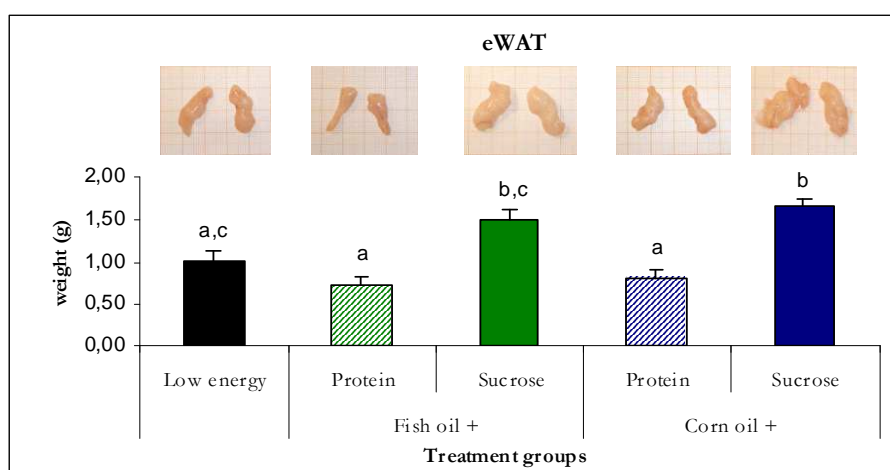


Figure 3.2: Weights (g) of eWAT from the 5 different treatment groups. The data are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the post-hoc Tukey's HSD test.

iWAT: The treatment groups receiving sucrose in the diet showed significantly higher iWAT weights than the rest of the groups receiving protein enriched- and low energy diet (figure 3.3). The fish oil + sucrose group had significantly higher iWAT weight compared to the control group ($P<0.003$), the fish oil + protein group ($P<0.0002$), and the corn oil + protein group ($P<0.0002$). The weight of iWAT from the corn oil + sucrose group was also significantly higher than the control group ($P<0.03$), the fish oil + protein group ($P<0.0006$) and the corn oil + protein group ($P<0.0006$).

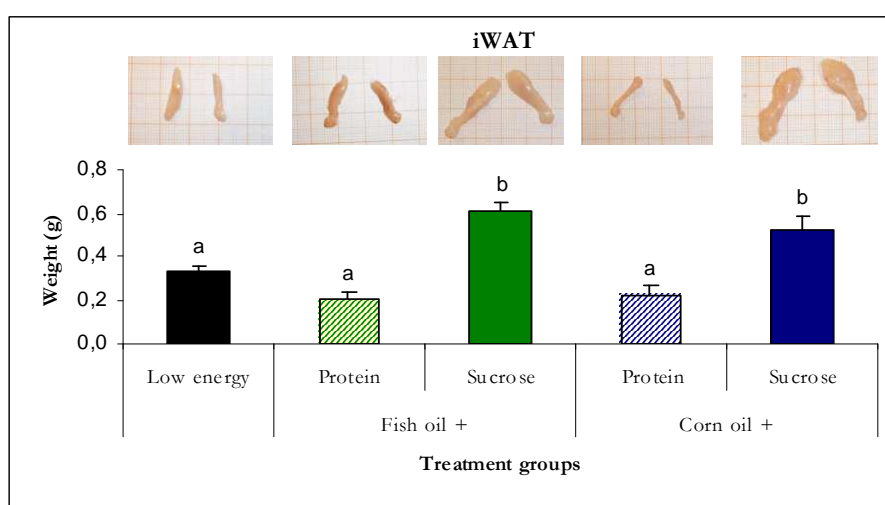


Figure 3.3: Weights (g) of iWAT from the 5 different treatment groups. The data are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P<0.05$, according to the post-hoc Tukey's HSD test.

rWAT: The weight of rWAT from the fish oil + sucrose group was significantly higher than the treatment groups receiving protein; fish oil + protein ($P<0.02$) and corn oil + protein ($P<0.03$) (figure 3.4). The control group and the corn oil + sucrose group did not reach any significant difference compared to the other treatment groups.

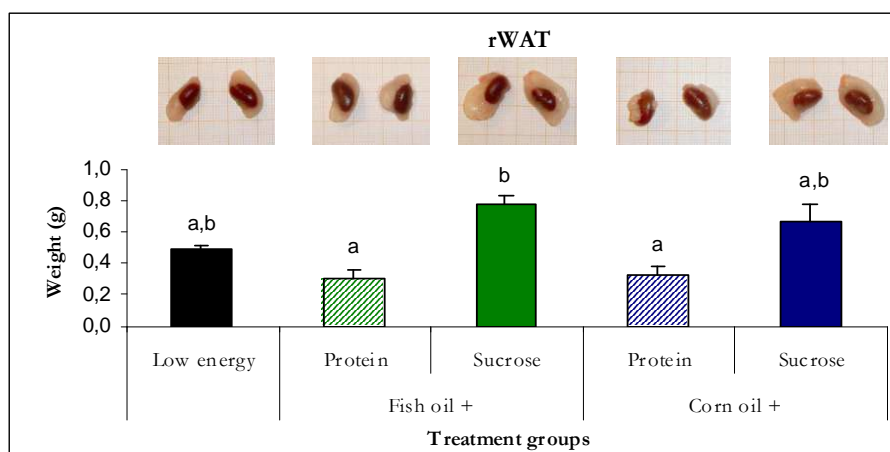


Figure 3.4: Weights (g) of rWAT from the 5 different treatment groups. The data are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the non-parametric test Kruskal-Wallis.

iBAT: The weight of iBAT for the fish oil + sucrose group was significantly higher than the treatment groups receiving protein in the diet; fish oil + protein ($P < 0.003$) and corn oil + protein ($P < 0.01$), but no statistical significance was reached compared to the control group and the corn oil + sucrose group (figure 3.5). The corn oil + sucrose group had also significantly higher weight of iBAT compared to the fish oil + protein group ($P < 0.02$) and the corn oil + protein group ($P < 0.04$).

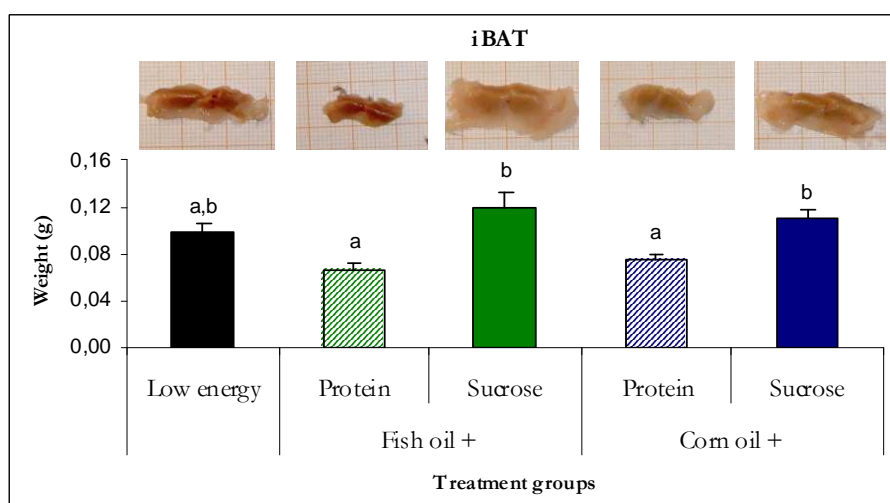


Figure 3.5: Weights (g) of iBAT from the 5 different treatment groups. The data are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the post-hoc Tukey's HSD test.

Liver: The weights of liver tissues were similarly increased for all the treatment groups, and no significant difference was reached (figure 3.6).

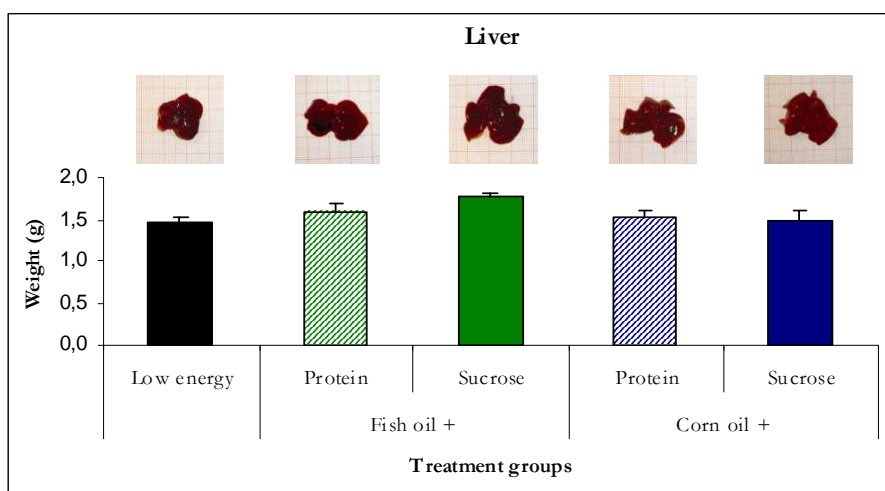


Figure 3.6: Weights (g) of livers from the 5 different treatment groups. The data are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the post-hoc Tukey's HSD test.

The results obtained from adipose tissue weights showed that the mice fed fish oil enriched with sucrose had significant higher adipose tissue weights compared to both the treatment groups receiving protein in the diet; fish oil + protein and corn oil + protein in eWAT, iWAT, rWAT and iBAT.

The mice's whole body was also photographed to assess the body size in general including the fat deposits. Figure 3.7 C and E suggest that mice fed diets with high sucrose have developed more body fat than the mice fed high-protein diets, which is positively correlated with the statistical analysis obtained from adipose tissue weights. Both mice from fish oil + sucrose- and corn oil + sucrose group showed clearly enlarged fat deposits compared with the control group fed low energy. Mice fed diets with high protein (figure 3.7 B and D), do not differ much in both body- and adipose deposits size from the control group, which also correlates well with the statistical analyses from adipose tissue weights.

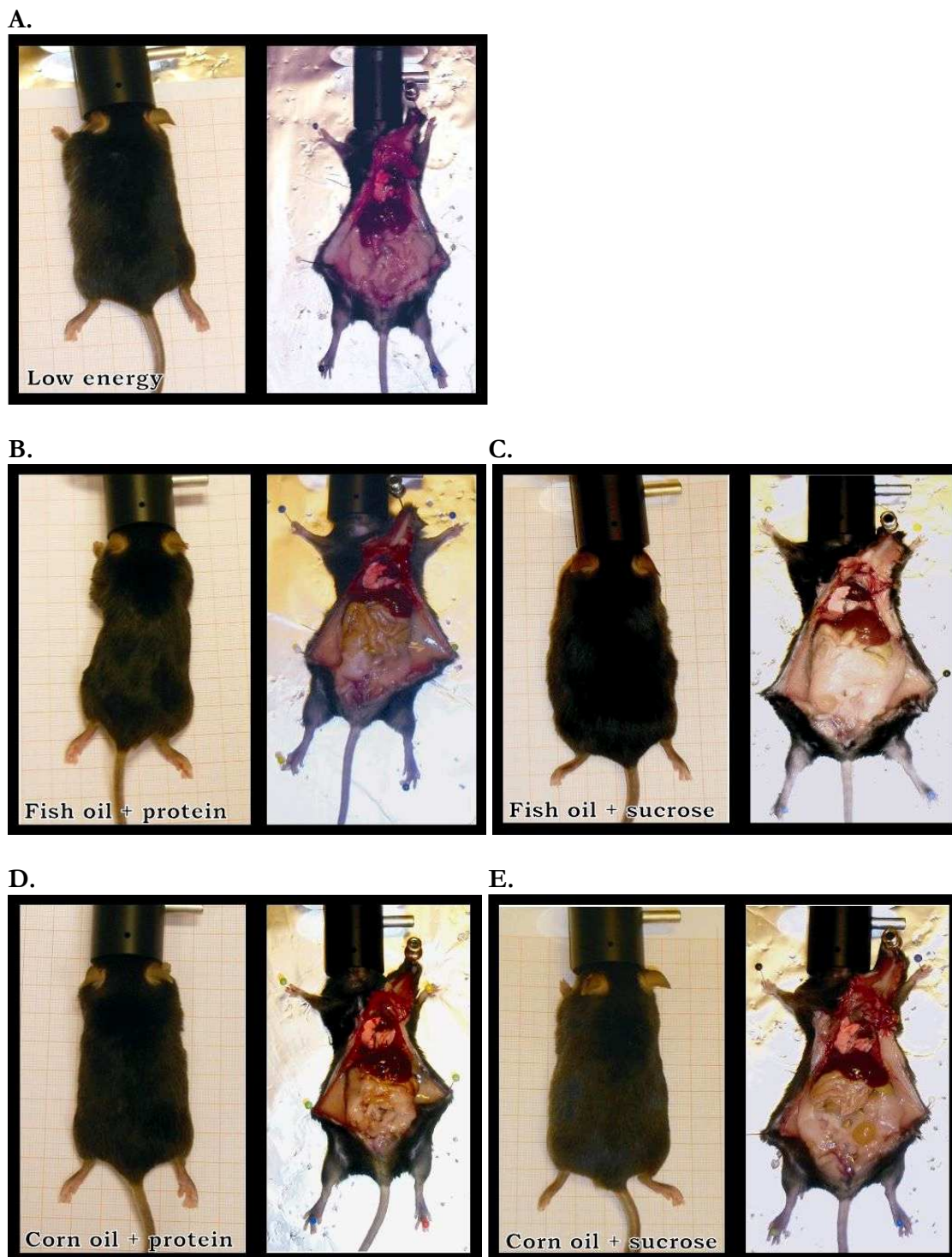


Figure 3.7: Photographs of representative mice from each treatment group taken after 63 days on feeding trial. The mice fed sucrose in the diet (C and E) appeared to have more body fat (obesigenic) compared to the mouse from the control group (A) and the mice fed protein in the diet (B and D).

3.1.3 Gene expression analysis - adipocyte differentiation

To investigate whether development of obesity had occurred as a result of hypertrophy or hyperplasia or both, the relative expression levels of some genes involved in adipocyte differentiation was measured (Figure 3.8, 3.9, 3.10). To ensure adequate RNA quality and integrity, the purified RNA from the tissues was analyzed using the Nanodrop ND-1000 and the Bioanalyzer (RNA 6000 Nano), respectively. The results obtained are shown in appendix VIII and IX.

PPAR- γ : There was no significant difference in gene expression level of PPAR- γ between the different treatment groups in iWAT and iBAT (figure 3.8). The relative expression level of PPAR- γ was significantly higher for the corn oil + protein group compared to the fish oil + protein group in eWAT ($P < 0.03$). There were no significant differences of expression levels for any of the groups comparing with the control group in eWAT. The control group receiving low energy had significantly higher relative expression level of PPAR- γ than the fish oil + protein and fish oil + sucrose group ($P < 0.03$, $P < 0.02$ respectively) in rWAT.

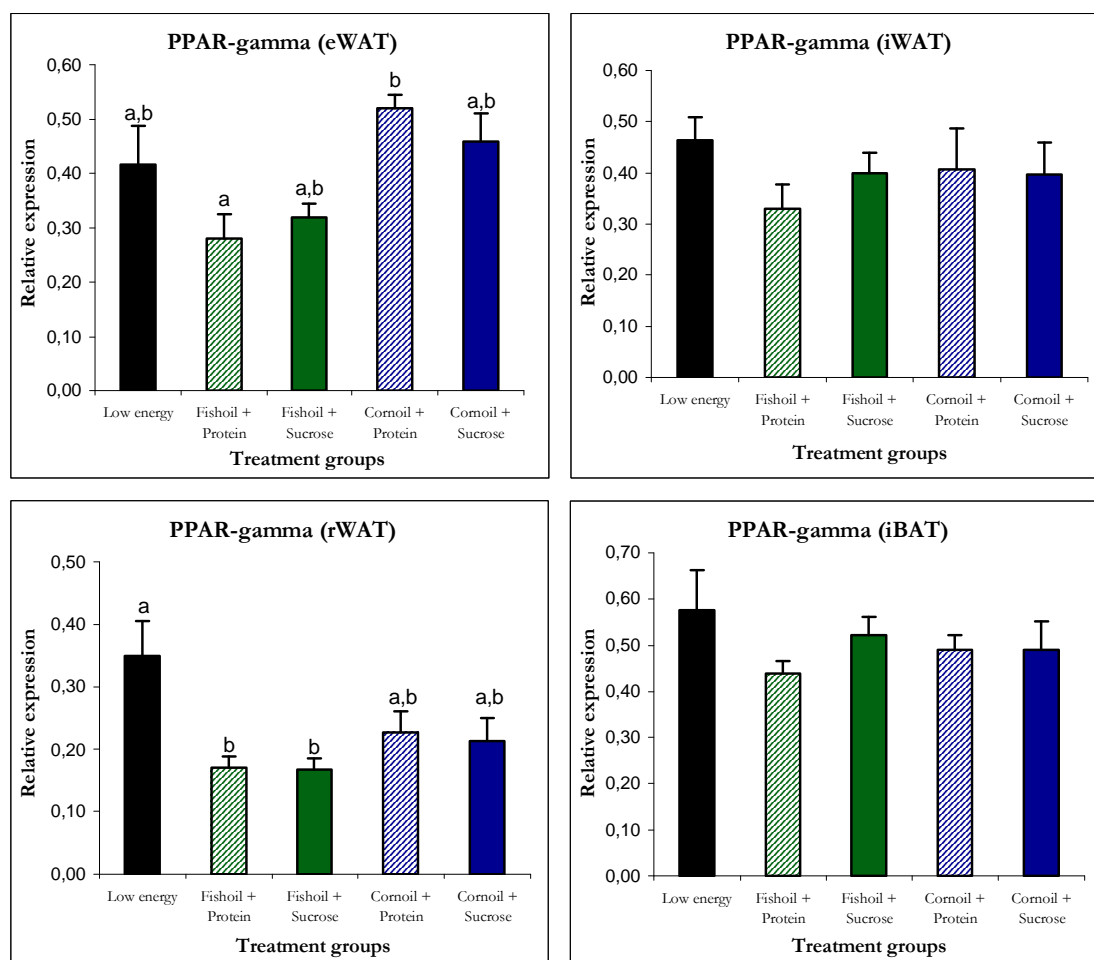


Figure 3.8: Relative expression levels of PPAR- γ for eWAT, iWAT, rWAT and iBAT. The results are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the post-hoc Tukey's HSD test (for eWAT) and the non-parametric test Kruskal-Wallis (for rWAT).

Pref-1: No significant differences were reached in gene expression level of Pref-1 between the different treatment groups in eWAT, iWAT and rWAT (figure 3.9). The expression level of Pref-1 in iBAT from the corn oil + protein group was significantly higher compared to the control group ($P < 0.05$).

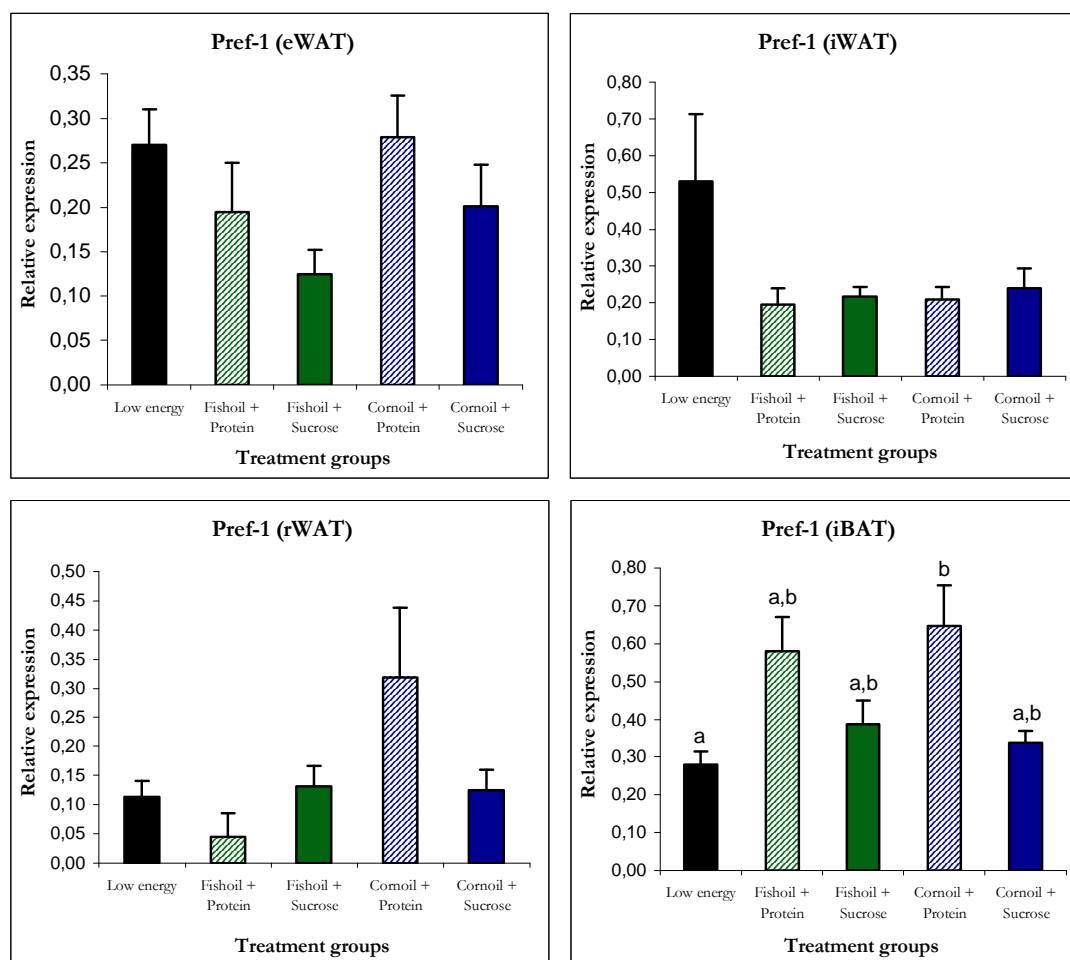


Figure 3.9: Relative expression levels of Pref-1 for eWAT, iWAT, rWAT and iBAT. The results are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the non-parametric test Kruskal-Wallis.

Adiponectin: The relative expression levels of adiponectin showed no significant difference between the 5 treatment groups in eWAT, iWAT and rWAT (figure 3.10). The fish oil + protein group had significantly higher expression level of adiponectin in iBAT compared to the corn oil + sucrose group ($P < 0.05$). There were no significant differences of expression levels for any of the groups comparing with the control group in iBAT.

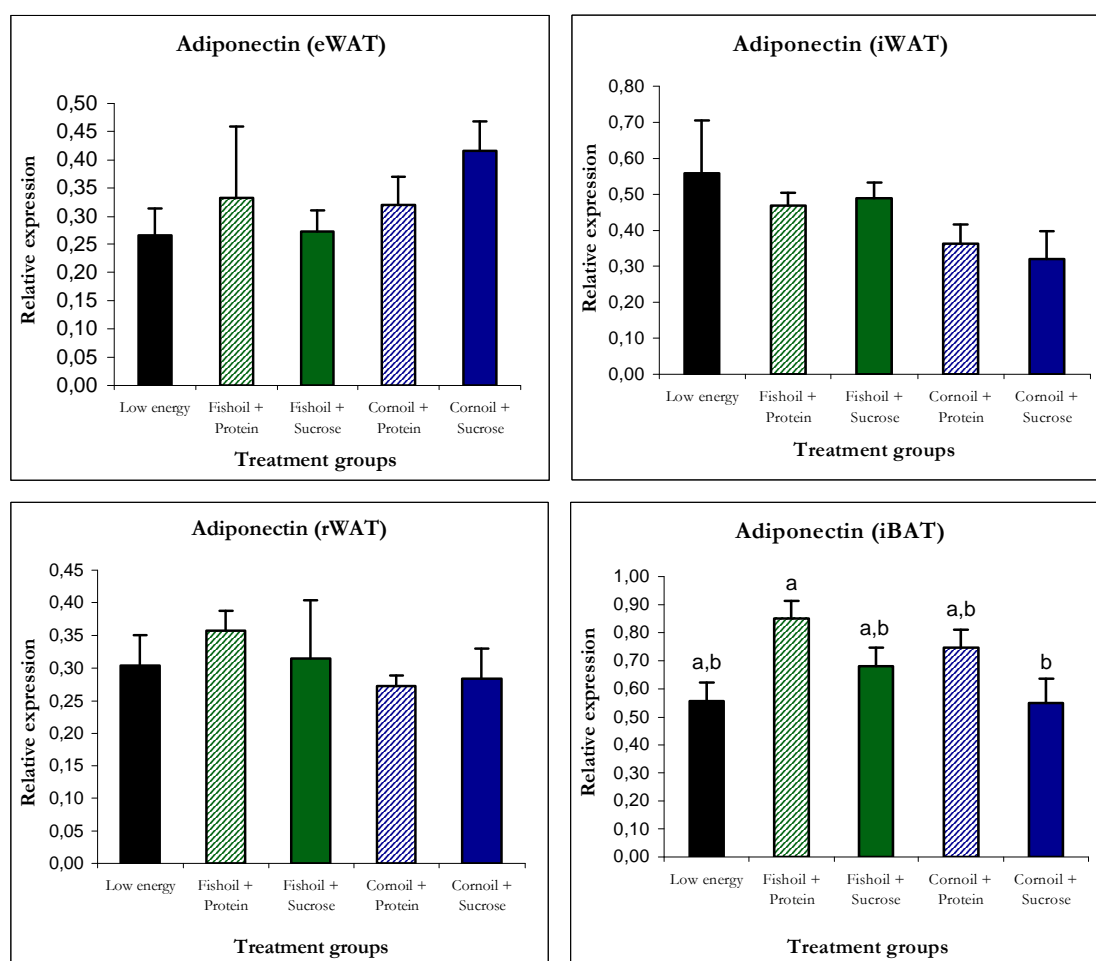


Figure 3.10: Relative expression levels of adiponectin for eWAT, iWAT, rWAT and iBAT. The results are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the non-parametric test Kruskal-Wallis.

PAI-1: No differences were found in the expression levels of PAI-1 between the different treatment groups in eWAT, rWAT and iBAT (figure 3.11). The only significant difference in relative expression level was in iWAT between the control group and the fish oil + protein group, where the control group had significantly higher expression of PAI-1 ($P < 0.04$).

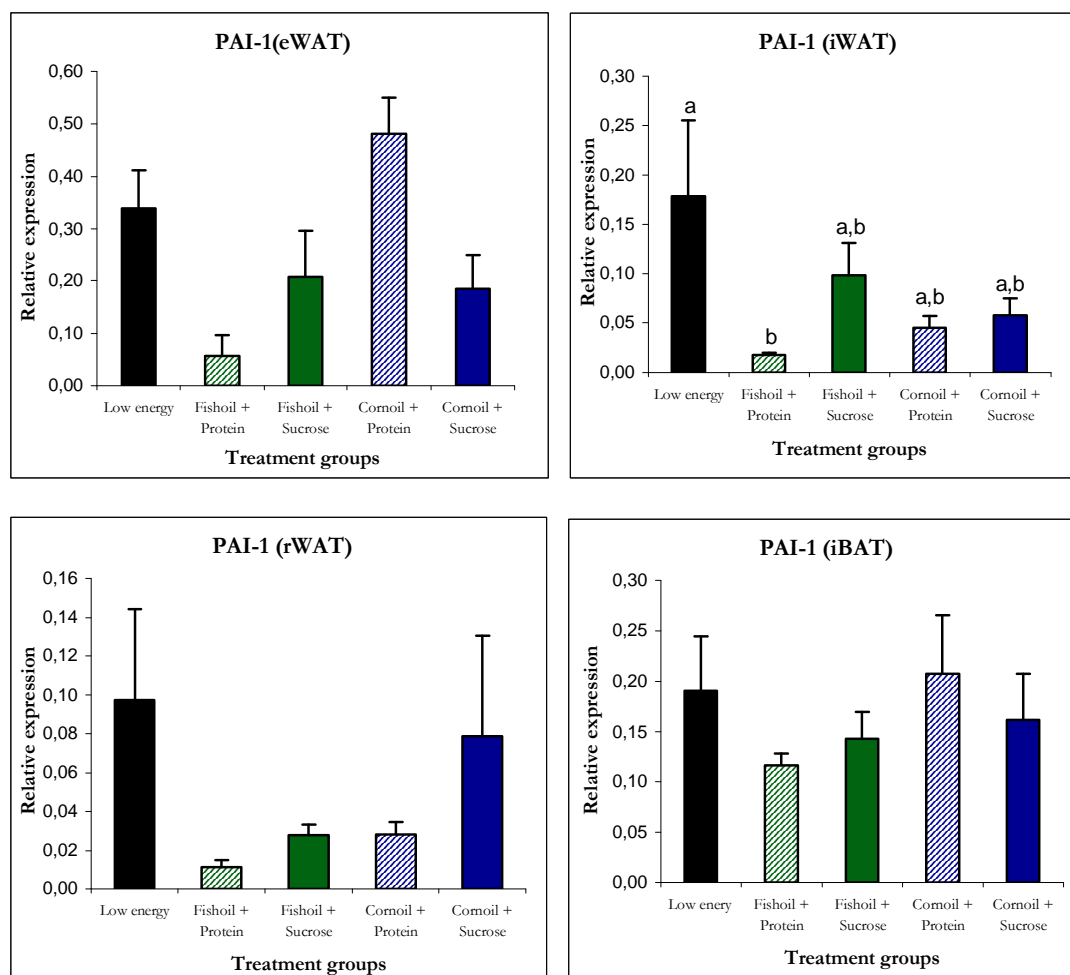


Figure 3.11: Relative expression levels of PAI-1 for eWAT, iWAT, rWAT and iBAT. The results are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the non-parametric test Kruskal-Wallis.

3.1.4 Histology

Histological sections were prepared to further investigate the obesity development of the different treatment groups. Pictures taken of eWAT and iWAT sections (figure 3.12 and 3.13) with the microscope Olympus camera, showed enlarged adipocyte size from the treatment groups receiving sucrose compared to the treatment groups receiving protein in

the diet. This pattern can especially be seen in iWAT sections (figure 3.13), where the adipocytes are clearly larger in group C and E than B and D. The adipocyte size of the protein groups (B and D) are also smaller compared to the control group A, receiving a low energy diet.

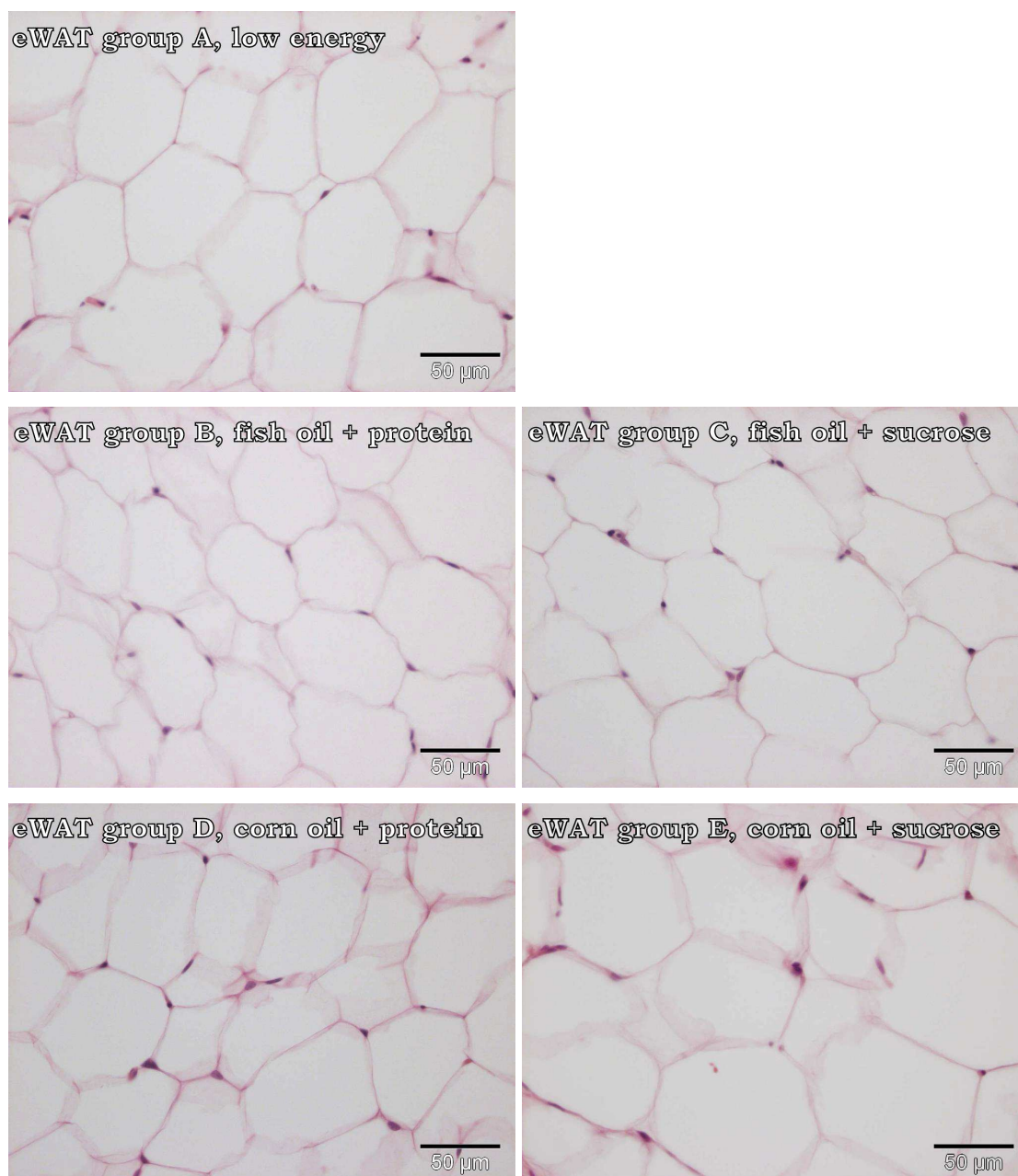


Figure 3.12: Microscopy pictures of paraffin-embedded sections of eWAT. The treatment groups C and E fed diets enriched with sucrose shows enlarged adipocytes compared with group A, B and D fed low energy or protein enriched diets. There are no crown-like structures localized in these sections. The sections were stained with H&E. The magnification was set at 400X, scale bar = 50 µm.

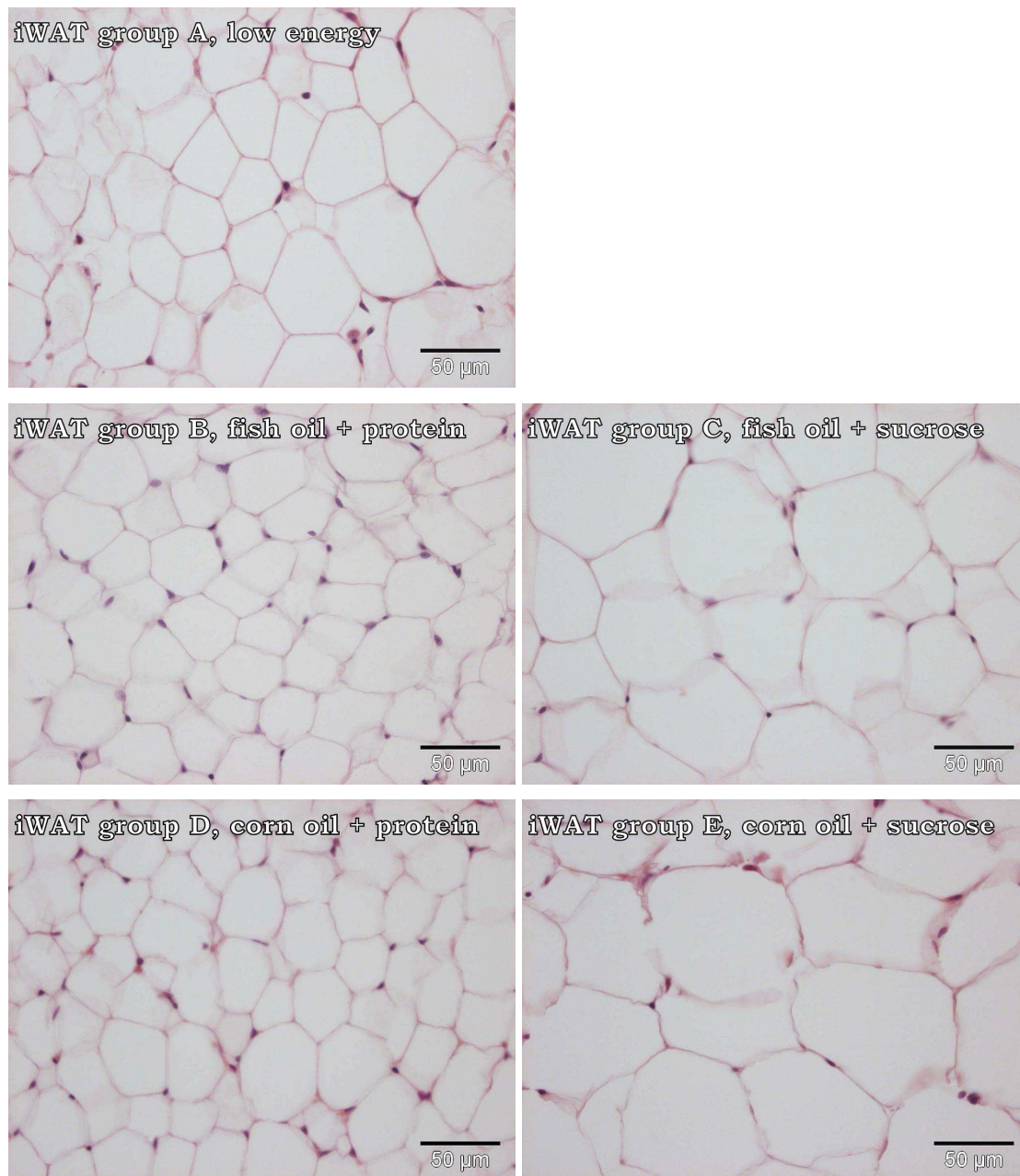


Figure 3.13: Microscopy pictures of paraffin-embedded iWAT sections. The adipocytes are clearly enlarged in the sections from mice fed a sucrose enriched diet (group C and E). The adipocyte size from the sections of mice fed protein enriched diets (group B and D) are smaller compared to the control group A receiving low energy. The sections were stained with H&E. The magnification was set at 400X, scale bar = 50 µm.

3.2 THE EFFECT OF THE DIFFERENT DIETS ON ADIPOSE TISSUE INFLAMMATION

After investigating the obesity development of the different treatment groups, a subsequent assessment of inflammation status was done by measuring gene expression levels of macrophage markers along with cytokines and chemokines.

3.2.1 *Gene expression analysis - macrophage markers*

Although no crown-like structures were seen in the sections, expressions of genes selectively expressed in macrophages were measured. The macrophage marks genes measured in this study were: F4/80 and CD68. The figure of relative expression levels of CD68 in iWAT is not shown, because of incomplete detection of the samples when performing qPCR.

F4/80: There were found no significant differences between the different treatment groups in relative expression levels of F4/80 in iWAT, rWAT and iBAT (figure 3.14). Statistical significance between the groups was only reached in eWAT, whereas the fish oil + sucrose group had significantly higher relative expression level than the control- ($P < 0.01$) and the fish oil + protein group ($P < 0.009$).

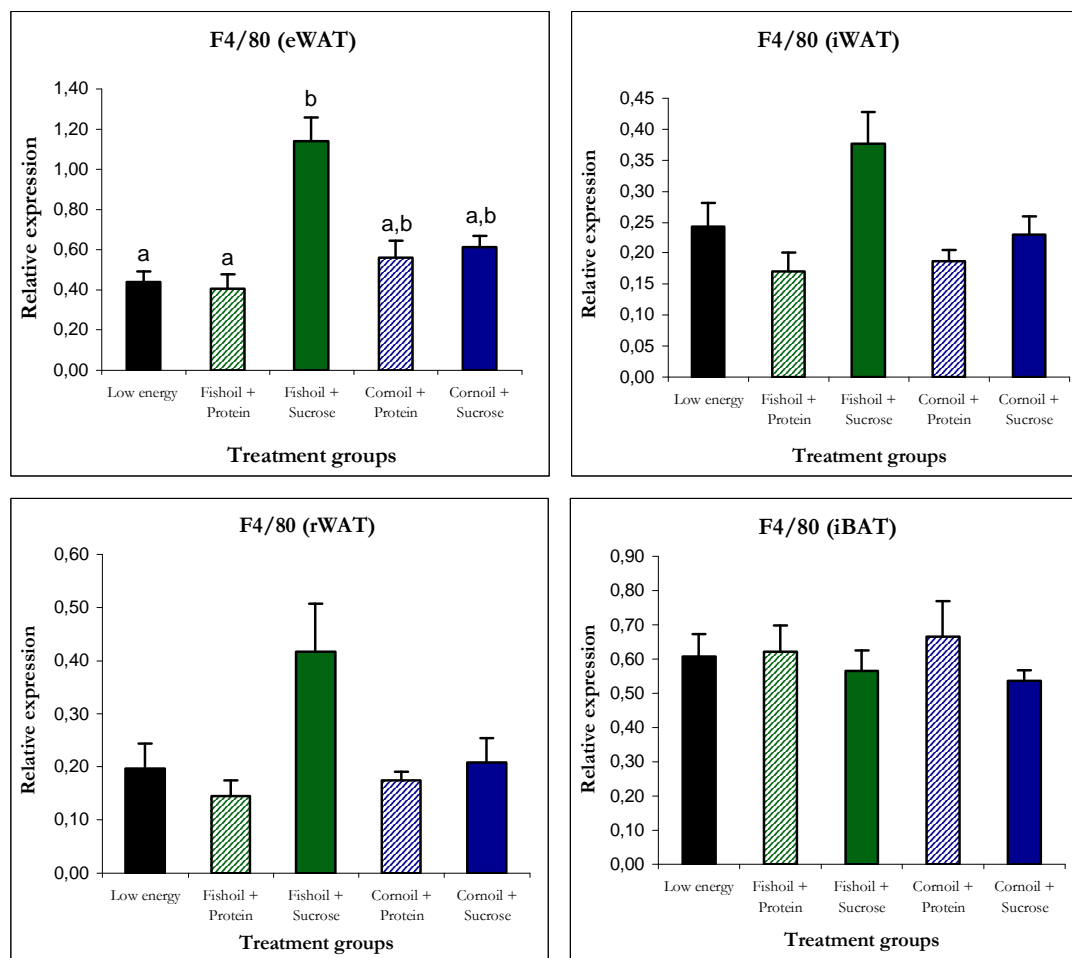


Figure 3.14: Relative expression levels of F4/80 for eWAT, iWAT, rWAT and iBAT. The results are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the non-parametric test Kruskal-Wallis.

CD68: Significant difference of relative expression level of CD68 between the different treatment groups was only reached in eWAT (figure 3.15). The fish oil + protein group had also significantly higher relative expression of CD68 compared to the control group ($P < 0.01$) and the corn oil + protein group ($P < 0.04$).

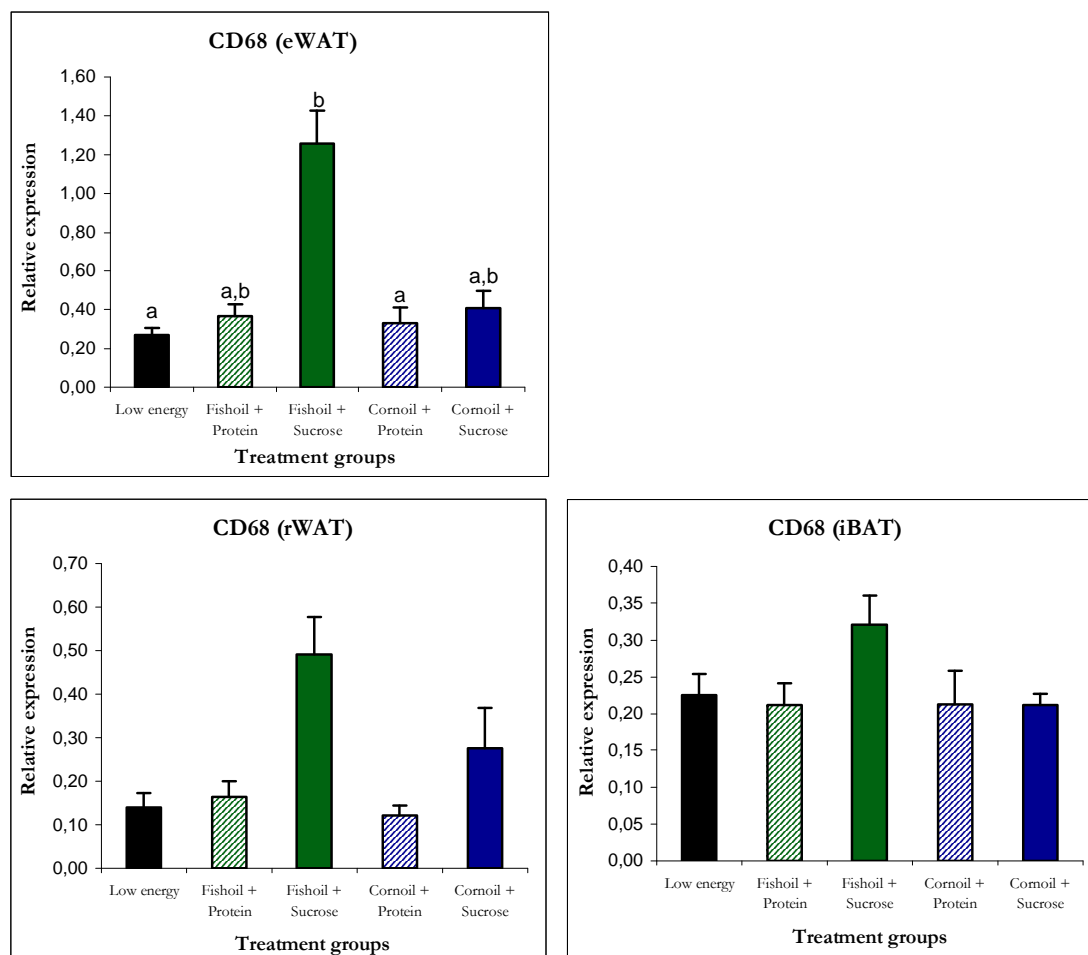


Figure 3.15: Relative expression levels of CD68 for eWAT, rWAT and iBAT. The results are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the non-parametric test Kruskal-Wallis.

3.2.2 Gene expression analysis – cytokines and chemokines

As mentioned earlier, adipose tissue is an endocrine organ. Thus, inflammatory cytokines such as IL-6, TNF-alpha and MCP-1 was measured by qPCR.

IL-6: Relative expression levels of IL-6 from the different treatment groups are shown in figure 3.16, where the control group and the fish oil + protein group had significant lower expression levels than the fish oil + sucrose group ($P < 0.009$, $P < 0.05$ respectively).

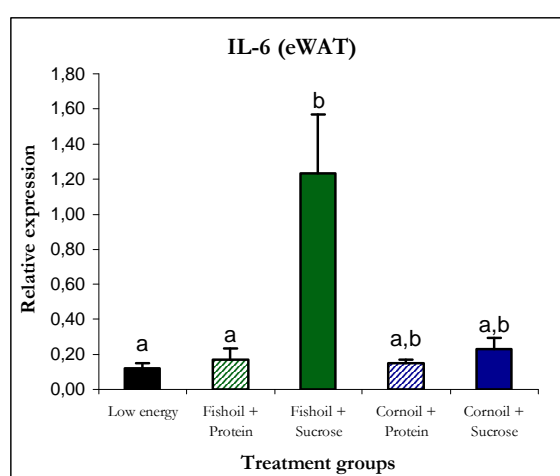


Figure 3.16: Relative expression levels of IL-6 for eWAT. (Figures of expression levels for the other tissues are not shown because of incomplete detection of the samples during quantitative real-time PCR run.) The result is presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the non-parametric test Kruskal-Wallis.

TNF- α : Figure 3.17 shows no significant differences between the different treatment groups of relative expression level of TNF- α in iWAT and rWAT. The expression level in eWAT was significantly higher for the fish oil + sucrose group compared to the control group, the fish oil + protein group and the corn oil + protein group ($P < 0.06$, $P < 0.0007$ and $P < 0.007$ respectively). The relative expression level of TNF- α in iBAT gave significant difference between the fish oil + sucrose group and the control group, where the control group had significantly lower expression levels ($P < 0.03$).

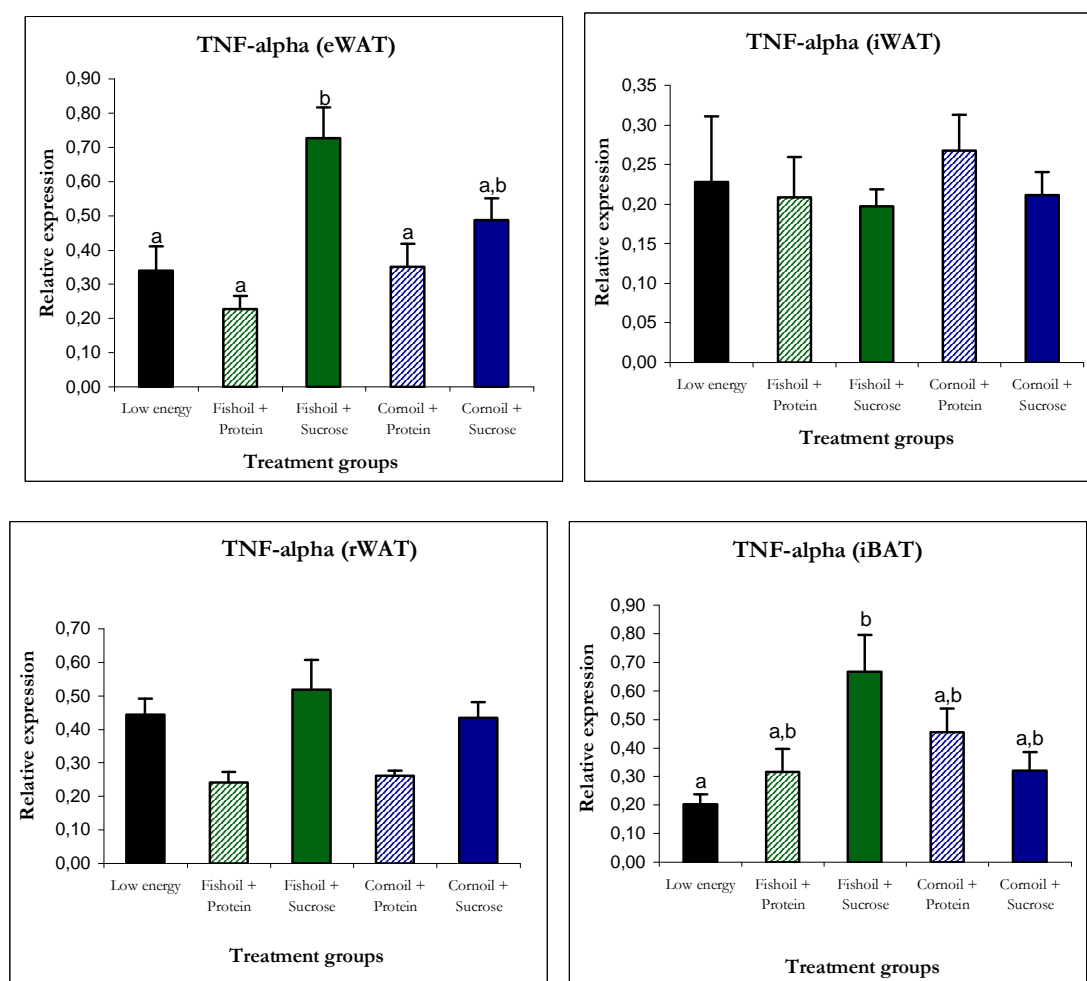


Figure 3.17: Relative expression levels of TNF- α for eWAT, iWAT, rWAT and iBAT. The results are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the post-hoc ANOVA Tukey's HSD test (for eWAT and rWAT) and the non-parametric test Kruskal-Wallis (for iWAT and iBAT).

MCP-1: The relative expression levels of MCP-1 in rWAT showed no significant difference between the different treatment groups (figure 3.18). There was no significant difference of expression level for any of the groups comparing with the control group in both eWAT and iWAT. The fish oil + sucrose group had significantly higher relative expression level of MCP-1 than the fish oil + protein group ($P < 0.004$) in eWAT. Relative expression level of MCP-1 in iWAT for the fish oil + sucrose group was also only significantly higher than the fish oil + protein group ($P < 0.03$). The only significant difference in relative MCP-1 expression in iBAT was reached between the fish oil + sucrose group and the control group, where the control group had significantly lower relative expression level ($P < 0.05$).

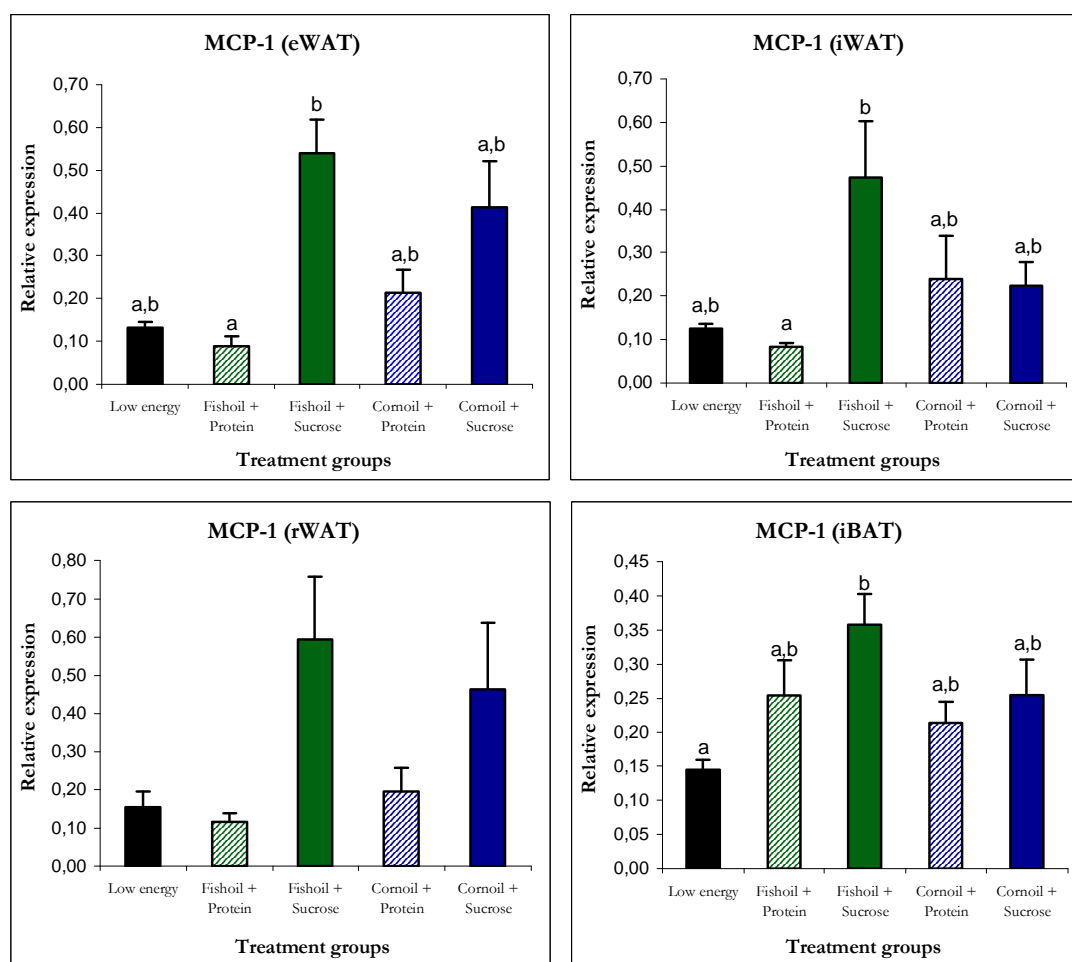


Figure 3.18: Relative expression levels of MCP-1 for eWAT, iWAT, rWAT and iBAT. The results are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the non-parametric test Kruskal-Wallis.

3.3 THE EFFECT OF THE DIFFERENT DIETS ON METABOLISM

Earlier studies in this laboratory have revealed that increasing the amount of protein to a corn oil enriched diet reduced the feed efficiency (Madsen, Pedersen *et al.*, 2008). Whether increasing the amount of protein to a diet enriched in fish oil, was however not shown.

3.3.1 Body weight gain and feed intake

As mentioned before, the body weight gain and feed intake data can be used as an assessment for the mice's contentment and development. The body weight gain for the treatment group receiving fish oil + sucrose was significantly higher than the groups receiving fish oil + protein ($P < 0.05$) and corn oil + protein ($P < 0.04$) (figure 3.19), but not significantly different compared to the control group. There was no significant difference in total feed intake in grams between the different treatment groups (figure 3.20). The total energy intake in kcal was however, significantly lower for the control group, due to a lower energy content in the diet compared to the fish oil + sucrose group ($P < 0.01$) and corn oil + sucrose group ($P < 0.004$) (figure 3.21 A). The results of total energy efficiency (bodyweight gain/Mcal consumed) for the fish oil + sucrose group was significantly higher than the treatment group receiving corn oil + protein ($P < 0.05$) (figure 3.21 B).

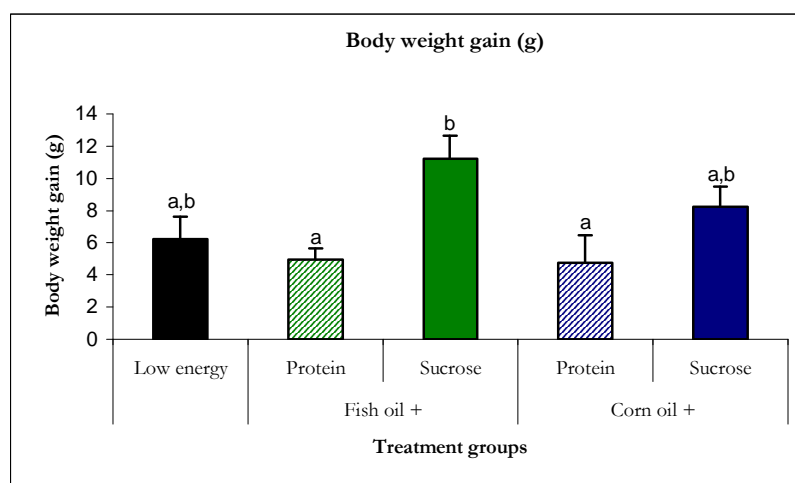


Figure 3.19: Body weight gain for the 5 different treatment groups after the feeding trial of 63 days. The data are presented as mean \pm SEM. The groups with different superscript letters are significantly different at $P < 0.05$, according to the post-hoc ANOVA Tukey's HSD test.

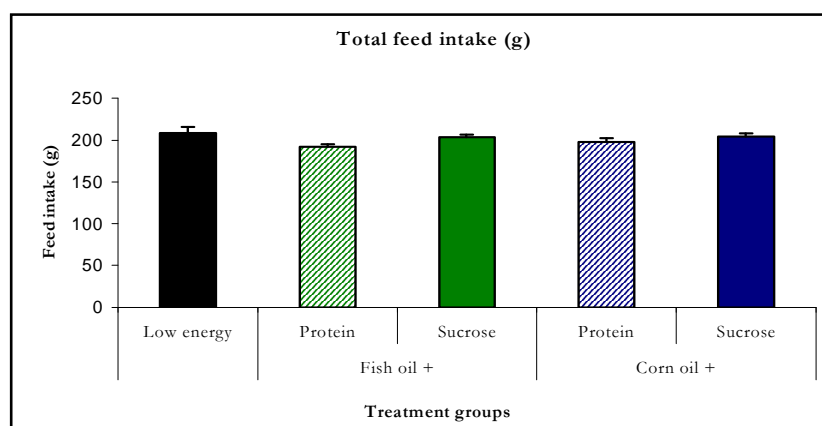


Figure 3.20: Total feed intake for the 5 different treatment groups during the feeding trial of 63 days. The data are presented as mean \pm SEM. No significant difference was found between the treatment groups according to the post-hoc ANOVA Tukey's HSD test.

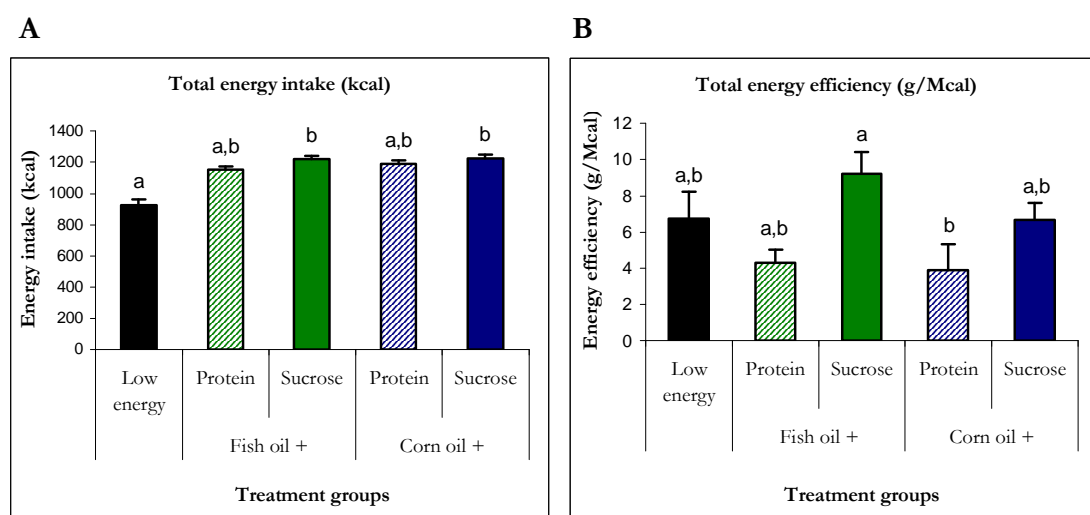


Figure 3.21: Total energy intake (kcal) and energy efficiency (g/Mcal) for the 5 different treatment groups during the feeding trial. The data are presented as mean \pm SEM. The groups with different superscript letters are significantly different at $P < 0.05$, according to the post-hoc ANOVA Tukey's HSD test (for total energy intake (kcal)) and the non-parametric test Kruskal Wallis (for total energy efficiency (g/Mcal)).

3.3.2 Determination of plasma parameters on the MAXMATTM PL

To investigate the insulin sensitivity of the mice fed the different diets, different plasma parameters were analysed.

There was found no significant differences in plasma concentrations of glucose, triacylglycerol (TAG), glycerol and free fatty acids (FFA) between the 5 different treatment groups (figure 3.22).

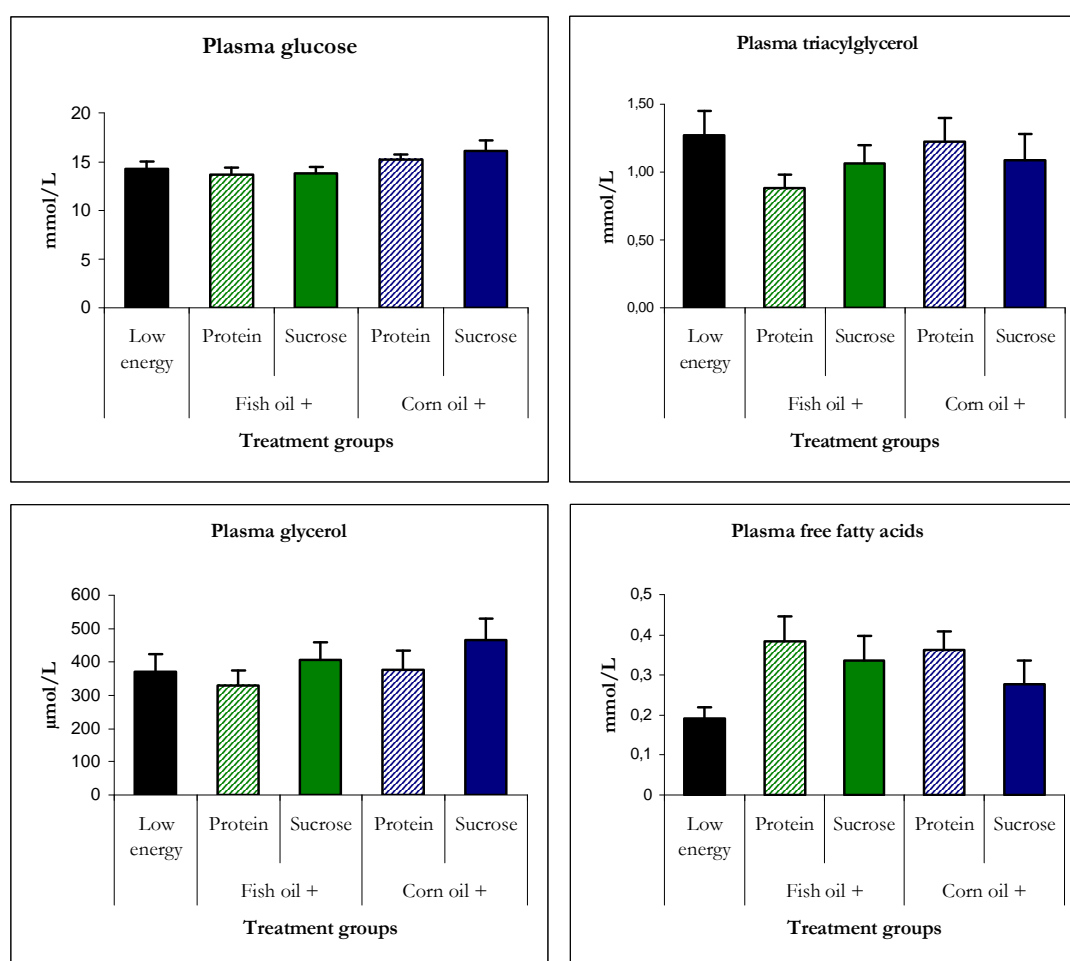


Figure 3.22: Plasma concentrations of glucose, triacylglycerol, glycerol and free fatty acids from the different treatment groups analysed on MAXMAT. Data are presented as mean \pm SEM, and statistical analysed by using post-hoc ANOVA Tukey's HSD test and the non- parametric test Kruskal-Wallis (for plasma free fatty acids), where $P < 0.05$ was considered as significant different.

There were also found no significant differences between the different treatment groups for plasma LDH and ALT analysis (figure 3.23). For plasma hydroxybutyrate significant lower concentration was reached for the control group compared to both of the groups with protein enriched diets (fish oil + protein $P < 0.0006$, corn oil + protein $P < 0.02$). The fish oil + protein group also had significantly higher concentration of plasma hydroxybutyrate compared to both of the groups receiving sucrose enriched diets (fish oil + sucrose $P < 0.004$, corn oil + sucrose $P < 0.007$).

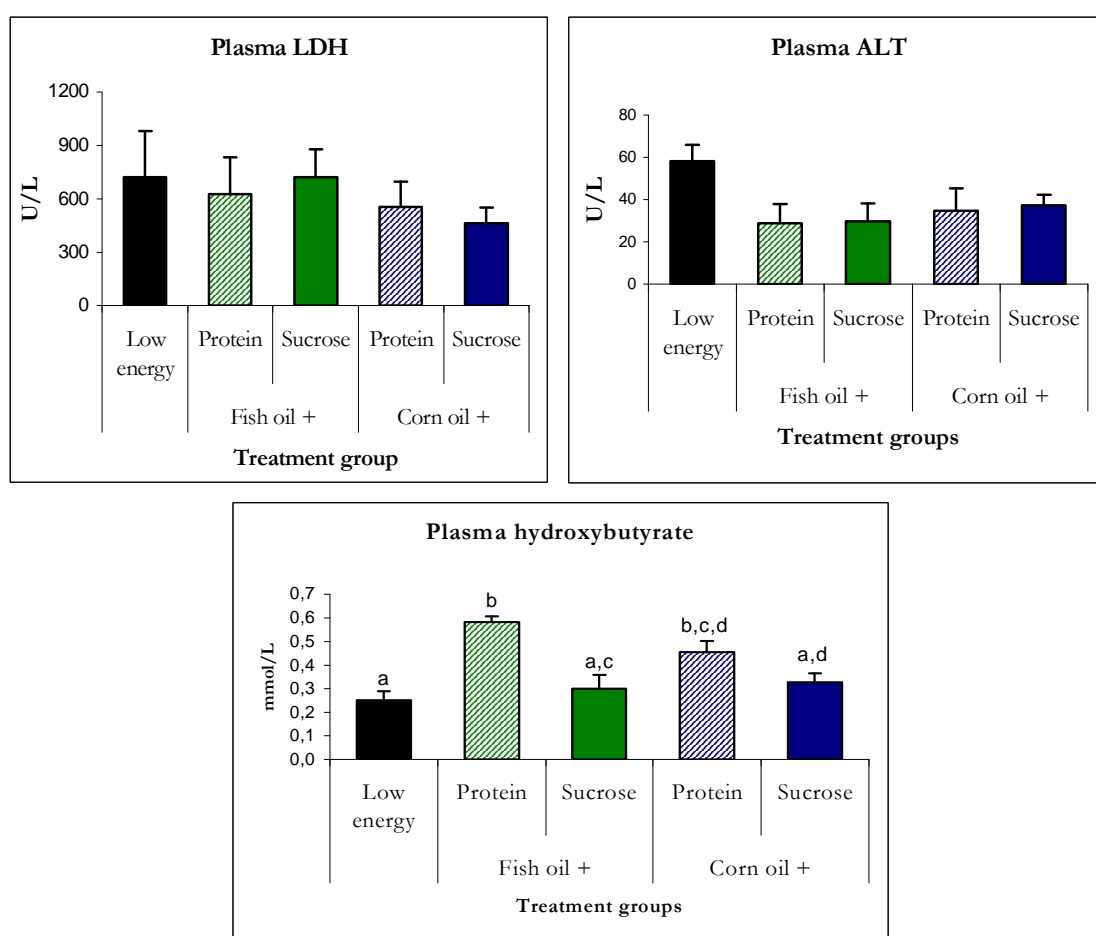


Figure 3.23: Plasma concentrations of LDH, ALT and hydroxybutyrate from the different treatment groups analysed on MAXMAT. Data are presented as mean \pm SEM, and statistical analysed by using and the non-parametric test Kruskal-Wallis (for LDH) and the post-hoc ANOVA Tukey's HSD test (for ALT and hydroxybutyrate). The groups with different superscript letters indicate significantly difference at $P < 0.05$.

3.3.3 Determination of insulin in plasma by ELISA

An ELISA kit was used in order to determine the insulin concentration in plasma from the different treatment groups. The results are shown in figure 3.22.

There was no significant difference in plasma insulin concentrations between the different treatment groups. However, the highest levels of insulin can be seen in the sucrose groups, where the corn oil + sucrose group has the highest concentration (684 ± 271 pmol/L).

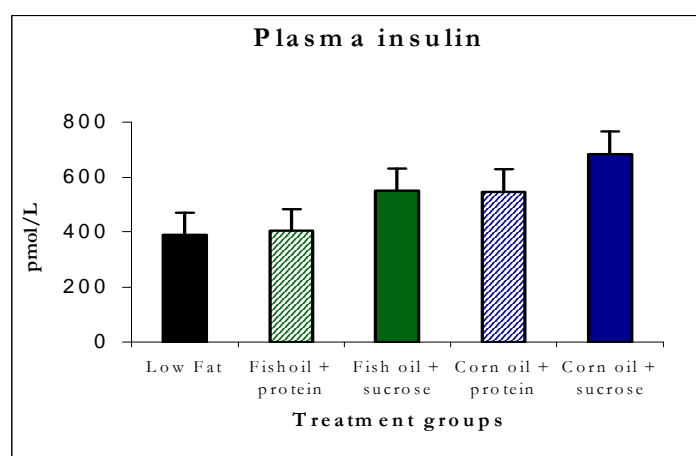


Figure 3.24: Plasma concentrations of insulin in pmol/L, determined by using ELISA kit. Data are presented as mean \pm SEM, and statistical analysed by using the non-parametric test Kruskal-Wallis, where $P < 0.05$ was considered as significant different.

3.3.4 Fatty acid composition by GLC

The results from the gene expression levels of the proinflammatory cytokines were rather surprising, and did not correspond to the previous studies where omega-3 in fish oil has been shown to have an anti-inflammatory effect (Itoh, Suganami *et al.*, 2007; Todoric, Loffler *et al.*, 2006). Thus a full fatty acid profile of the diets (appendix I) and RBC along with liver and eWAT was performed by GLC. The different compositions of selected Σ fatty acids of interest (given as mg fatty acid/g sample and % of total fatty acids) for this study are listed in table 3.1, 3.2, 3.3, 3.4, 3.5 and 3.6.

RBC: The fatty acid composition in RBC can be reflected on the amount of fish oil ingested from the diet over time according to a human pilot study by De Groote *et.al.* (2008) (De Groote, De Laporte et al., 2008). As expected for this study, the \sum n-3 comprising among other fatty acids; ALA (18:3n-3), EPA (20:5n-3) and DHA (22:6n-3) was significantly higher in the groups fed fish oil compared to the groups receiving corn oil ($P < 0.05$) (table 3.1 and 3.2). The groups receiving diets rich in n-6 had had significantly higher levels of LA (18:2n-6) and AA (20:4n-6) in the RBC ($P < 0.004$). The fatty acid composition in the different diets (appendix I) confirms the higher amounts of n-3 fatty acids in the fish oil enriched diets as well as the higher amounts of n-6 fatty acids in the corn oil enriched diets.

Table 3.1: Absolute fatty acid composition in RBC

RBC Fatty acid (mg fatty acid/g sample)	Treatment groups				
	Low energy (n=6)	Fish oil + protein (n=5)	Fish oil + sucrose (n=5)	Corn oil + protein (n=6)	Corn oil + sucrose (n=6)
\sum saturated	1.28 \pm 0.02	1.17 \pm 0.02	1.24 \pm 0.03	1.21 \pm 0.04	1.31 \pm 0.05
\sum monounsaturated	0.49 \pm 0.01 ^a	0.35 \pm 0.01 ^b	0.34 \pm 0.01 ^b	0.40 \pm 0.01 ^c	0.41 \pm 0.01 ^c
\sum polyenes	1.40 \pm 0.02 ^a	1.19 \pm 0.02 ^b	1.28 \pm 0.04 ^{ab}	1.45 \pm 0.04 ^{ac}	1.53 \pm 0.05 ^{ac}
\sum n-3	0.25 \pm 0.00 ^{ab,c}	0.72 \pm 0.02 ^{ac}	0.79 \pm 0.02 ^a	0.11 \pm 0.00 ^b	0.21 \pm 0.01 ^{bc}
\sum n-6	1.14 \pm 0.02 ^{ab}	0.47 \pm 0.01 ^a	0.49 \pm 0.01 ^a	1.34 \pm 0.04 ^b	1.32 \pm 0.05 ^b
n-3/n-6	0.22 \pm 0.00 ^{ab,c}	1.51 \pm 0.02 ^{ac}	1.62 \pm 0.01 ^a	0.08 \pm 0.00 ^b	0.16 \pm 0.00 ^{bc}

The data is presented as mg fatty acid/g sample, mean \pm SEM. The small letters indicate significant difference between the groups according to the post-hoc ANOVA Tukey's HSD test (for \sum monoenes and \sum polyenes) and the non-parametric test Kruskal Wallis. Differences were deemed statistical significant when $P < 0.05$.

Table 3.2: Relative fatty acid composition in RBC.

RBC Fatty acid (% of total fatty acids)	Treatment groups				
	Low energy (n=6)	Fish oil + protein (n=5)	Fish oil + sucrose (n=5)	Corn oil + protein (n=6)	Corn oil + sucrose (n=6)
\sum n-3	7.31 \pm 0.12 ^{ab,c}	24.88 \pm 0.26 ^{ac}	25.97 \pm 0.18 ^a	3.22 \pm 0.05 ^b	5.97 \pm 0.09 ^{bc}
\sum n-6	33.54 \pm 0.15 ^{ab,c}	16.52 \pm 0.15 ^{ac}	16.08 \pm 0.09 ^a	39.94 \pm 0.26 ^b	37.43 \pm 0.37 ^{bc}
n-3/n-6	0.22 \pm 0.00 ^{ab,c}	1.51 \pm 0.02 ^{ac}	1.62 \pm 0.01 ^a	0.08 \pm 0.00 ^b	0.16 \pm 0.00 ^{bc}

The data is presented as % of total fatty acids, mean \pm SEM. The small letters indicate significant difference between the groups according to the non-parametric test Kruskal Wallis. Differences were deemed statistical significant when $P < 0.05$.

Liver: There were found higher amount of saturated and monounsaturated in the liver of treatment groups fed sucrose compared to the ones fed protein (table 3.3). Although no significant differences were found between the groups mentioned. The accumulation of n-3 fatty acids in the liver was higher for the fish oil group receiving sucrose compared to the fish oil + protein group. The amount of n-6 fatty acids was also higher in the liver for the treatment group fed corn oil + sucrose, compared to the corn oil + protein group. Also here the there were found no significant differences between the corn oil groups.

Table 3.4 shows the % of total fatty acid, where the amount of n-3 fatty acids is significantly higher for the fish oil groups compared to the corn oil groups ($P < 0.05$). The amount of n-6 fatty acids is higher for the corn oil groups compared to the rest of the treatment groups, although the significant differences was reached between the corn oil + protein group and the fish oil + protein- and fish oil + sucrose group ($P < 0.006$, $P < 0.00008$ respectively). Significantly higher amount of n-6 fatty acids in the liver for the corn oil + sucrose group compared to the fish oil + sucrose group were also found ($P < 0.009$).

Table 3.3: Absolute fatty acid composition in liver.

Liver Fatty acid (mg fatty acid/g sample)	Treatment groups				
	Low energy (n=6)	Fish oil + protein (n=5)	Fish oil + sucrose (n=5)	Corn oil + protein (n=6)	Corn oil + sucrose (n=6)
\sum saturated	14.69 \pm 1.17 ^{a,b}	12.15 \pm 0.83 ^a	15.06 \pm 0.50 ^{a,b}	13.19 \pm 0.68 ^{a,b}	21.02 \pm 3.05 ^b
\sum monounsaturated	13.44 \pm 1.64 ^{a,c}	4.16 \pm 0.48 ^b	6.23 \pm 1.14 ^{a,b,c}	7.34 \pm 0.85 ^{a,b,c}	19.03 \pm 3.99 ^c
\sum polyenes	16.50 \pm 1.40 ^a	17.67 \pm 1.17 ^a	19.43 \pm 0.86 ^{a,b}	20.19 \pm 1.21 ^{a,b}	29.33 \pm 2.88 ^b
\sum n-3	3.24 \pm 0.27 ^{a,b}	10.83 \pm 0.79 ^a	12.94 \pm 0.56 ^a	1.72 \pm 0.11 ^b	3.28 \pm 0.15 ^{a,b}
\sum n-6	13.26 \pm 1.14 ^{a,b}	6.84 \pm 0.42 ^a	6.49 \pm 0.32 ^a	18.47 \pm 1.13 ^b	26.05 \pm 2.77 ^b
n-3/n-6	0.25 \pm 0.01 ^{a,b,c}	1.58 \pm 0.06 ^{a,c}	2.00 \pm 0.03 ^a	0.09 \pm 0.01 ^b	0.13 \pm 0.01 ^{b,c}

The data is presented as mg fatty acid/g sample, mean \pm SEM. The small letters indicate significant difference between the groups according to the non-parametric test Kruskal Wallis. Differences were deemed statistical significant when $P < 0.05$.

Table 3.4: Relative fatty acid composition in liver.

Liver Fatty acid (% of total fatty acids)	Treatment groups				
	Low energy (n=6)	Fish oil + protein (n=5)	Fish oil + sucrose (n=5)	Corn oil + protein (n=6)	Corn oil + sucrose (n=6)
∑ n-3	7.25 ± 0.45 ^{ab}	31.34 ± 0.39 ^a	31.41 ± 1.37 ^a	4.10 ± 0.28 ^b	4.97 ± 0.53 ^b
∑ n-6	29.43 ± 1.18 ^{ab,c}	19.90 ± 0.55 ^{a,c}	15.78 ± 0.88 ^a	43.70 ± 0.73 ^b	37.69 ± 1.38 ^{b,c}
n-3/n-6	0.25 ± 0.01 ^{ab,c}	1.58 ± 0.06 ^{a,c}	2.00 ± 0.03 ^a	0.09 ± 0.01 ^b	0.13 ± 0.01 ^{b,c}

The data is presented as % of total fatty acids, mean ± SEM. The small letters indicate significant difference between the groups according to the non-parametric test Kruskal Wallis. Differences were deemed statistical significant when $P < 0.05$.

eWAT: Measurements of the fatty acid profile in the adipose tissue is also of interest, since a large amount of sucrose in the diet can promote the *de novo* fatty acid synthesis (Chong, Fielding *et al.*, 2007), which can lead to higher levels of saturated and monounsaturated fatty acids in the adipose tissue. The combination of fish oil and sucrose in the diet, might affect the level of n-3 fatty acids in the adipose tissue, not being as high as expected. The amount of saturated and monounsaturated fatty acids are quantitative higher for the treatment groups receiving sucrose compared to the ones receiving protein with the same type of fat (table 3.5). But both of the fish oil groups and corn oil groups were not significantly different from each other. Compared to the saturated and monounsaturated fatty acids in the RBC, showed no significant difference between the treatment groups receiving protein or sucrose (table 3.1). The saturated and monounsaturated fatty acids in the diet (appendix I, table A.1) did not show a remarkable difference between the protein and sucrose groups either.

Another possible effect of the sucrose in the diet is to conduct more fat from the diet directly on to the adipose tissue. This suggestion is supported by data shown in table 3.5 demonstrating that the amount of n-6 is higher in eWAT for the corn oil + sucrose group compared to the corn oil + protein group. Likewise, the amount of n-3 fatty acids is higher in eWAT from the fish oil + sucrose group compared to the fish oil + protein group. Table 3.6 also shows a higher accumulation of n-3 fatty acids in eWAT for the groups receiving fish oil in the diet compared to the ones receiving corn oil diets. The same pattern are shown for the corn oil groups, who have significantly higher levels of n-6 fatty acids in the eWAT ($P < 0.05$).

Table 3.5: Absolute fatty acid composition in eWAT.

eWAT Fatty acid (mg fatty acid/g sample)	Treatment groups				
	Low energy (n=6)	Fish oil + protein (n=5)	Fish oil + sucrose (n=5)	Corn oil + protein (n=6)	Corn oil + sucrose (n=6)
\sum saturated	198.13 \pm 6.16 ^{a,c}	208.37 \pm 25.12 ^{a,b}	250.14 \pm 8.27 ^b	120.14 \pm 7.43 ^c	155.20 \pm 8.06 ^c
\sum monounsaturated	371.74 \pm 8.72 ^a	267.90 \pm 28.98 ^b	269.15 \pm 6.45 ^b	278.97 \pm 11.74 ^b	305.22 \pm 6.64 ^b
\sum polyenes	295.18 \pm 2.15 ^a	270.59 \pm 32.23 ^{a,b}	308.11 \pm 9.29 ^{a,b}	340.60 \pm 24.01 ^{a,b}	374.76 \pm 14.29 ^b
\sum n-3	18.16 \pm 0.47 ^{a,b,c}	87.07 \pm 14.48 ^{a,c}	114.89 \pm 7.27 ^a	6.25 \pm 0.54 ^b	8.68 \pm 0.32 ^{b,c}
\sum n-6	277.03 \pm 1.91 ^{a,b}	180.78 \pm 19.22 ^a	190.09 \pm 3.77 ^a	334.35 \pm 23.54 ^b	366.08 \pm 14.04 ^b
n-3/n-6	0.07 \pm 0.00 ^{a,b,c}	0.47 \pm 0.05 ^{a,c}	0.60 \pm 0.04 ^a	0.02 \pm 0.00 ^b	0.02 \pm 0.00 ^{b,c}

The data is presented as mg fatty acid/g sample, mean \pm SEM. The small letters indicate significant difference between the groups according to the post-hoc ANOVA Tukey's HSD test (for \sum saturated and \sum monoenes) and the non-parametric test Kruskal Wallis. Differences were deemed statistical significant when $P < 0.05$.

Table 3.6: Relative fatty acid composition in eWAT.

eWAT Fatty acid (% of total fatty acids)	Treatment groups				
	Low energy (n=6)	Fish oil + protein (n=5)	Fish oil + sucrose (n=5)	Corn oil + protein (n=6)	Corn oil + sucrose (n=6)
\sum n-3	2.08 \pm 0.04 ^{a,b,c}	11.20 \pm 0.98 ^{a,c}	13.63 \pm 0.64 ^a	0.83 \pm 0.04 ^b	1.03 \pm 0.02 ^{b,c}
\sum n-6	31.85 \pm 0.60 ^{a,b}	23.91 \pm 0.66 ^a	22.64 \pm 0.50 ^a	44.62 \pm 0.71 ^b	43.52 \pm 0.37 ^b
n-3/n-6	0.07 \pm 0.00 ^{a,b,c}	0.47 \pm 0.05 ^{a,c}	0.60 \pm 0.04 ^a	0.02 \pm 0.00 ^b	0.02 \pm 0.00 ^{b,c}

The data is presented as % of total fatty acids, mean \pm SEM. The small letters indicate significant difference between the groups according to the non-parametric test Kruskal Wallis. Differences were deemed statistical significant when $P < 0.05$.

3.3.5 Gene expression analysis - metabolism

Both SREBP-1c and SCD1 are important genes regulating the metabolism of glucose, triglycerides and fatty acids (Haluzik, Colombo *et al.*, 2004). The genes were measured in liver tissue by qPCR, and the relative gene expression levels are presented in figure 3.23. UCP-1 was also measured in the different adipose tissue (eWAT, iWAT, rWAT and iBAT). UCP-1 is known to exclusively be found in iBAT and play an important role in adaptative thermogenesis and energy expenditure (Lodhi and Semenkovich, 2009; Nedergaard, Golozoubova *et al.*, 2001).

SREBP-1c: The relative expression level of SREBP-1c in liver tissue was significant higher in the control group compared to the fish oil + protein group ($P < 0.004$).

SCD1: The control group showed significant higher relative expression levels of SCD1 in liver compared to the fish oil + protein- and fish oil + sucrose group ($P < 0.03$, $P < 0.004$ respectively). The fish oil + sucrose group also showed significantly lower levels of SCD1 compared to the corn oil + sucrose group ($P < 0.03$).

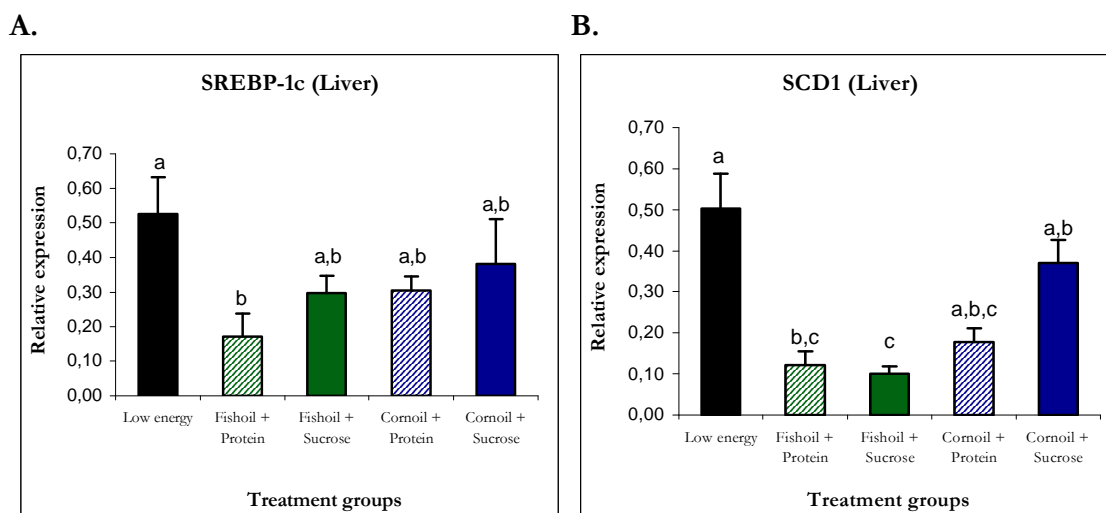


Figure 3.25: Relative expression levels of SREBP-1c (A) and SCD1 (B) in liver for the different treatment groups. Data is presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the non-parametric test Kruskal-Wallis.

UCP-1: The relative expression levels of UCP-1 were in general very low in all of the adipose tissues. However, the highest relative expression level was found in iBAT (figure 3.24). There were no significant differences between the treatment groups regarding the relative expression levels of UCP-1 in eWAT, iWAT and iBAT. The only significant difference was reached in rWAT, whereas the fish oil + sucrose group had significantly higher levels of UCP-1 compared to fish oil + protein- and the corn oil + protein group ($P < 0.008$, $P < 0.02$ respectively). There was no significant difference in expression level of UCP-1 between the control group and the rest of the treatment groups.

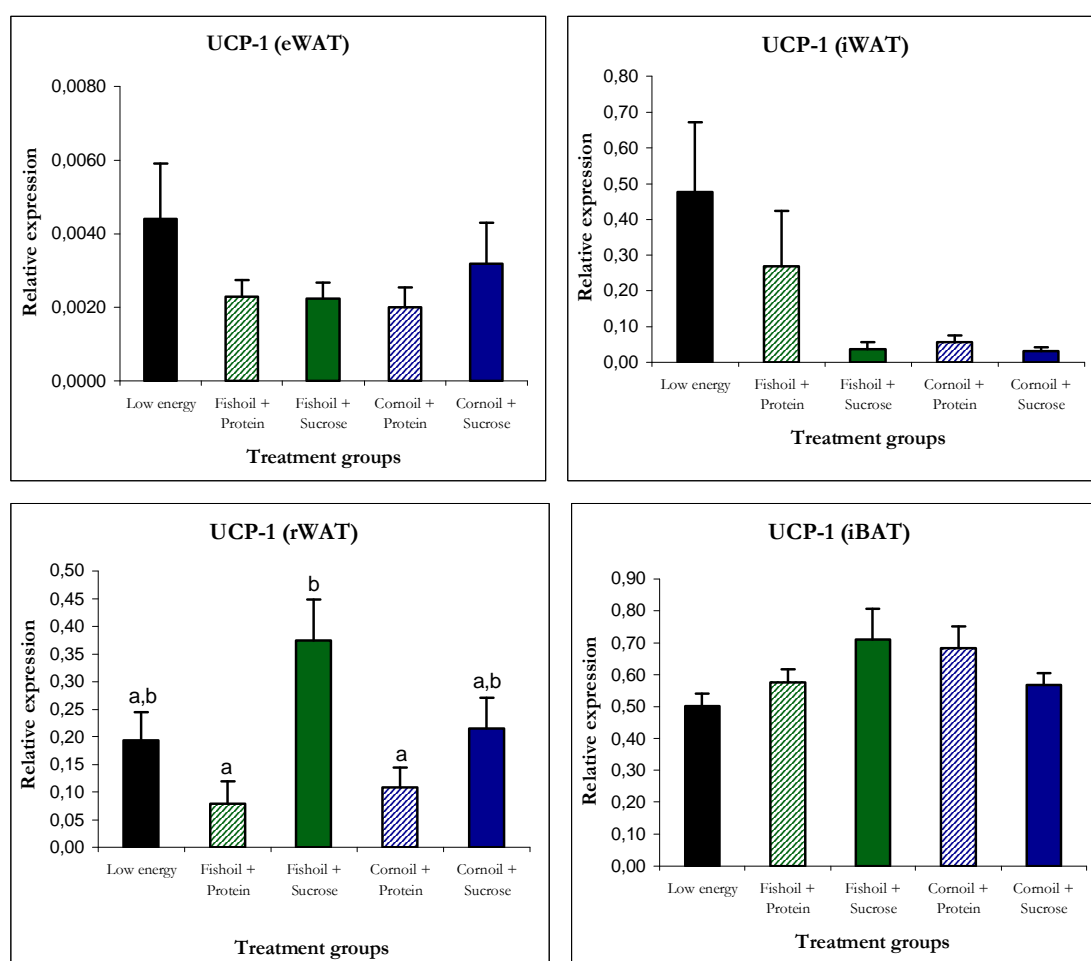


Figure 3.26: Relative expression levels of UCP-1 for eWAT, iWAT, rWAT and iBAT. The results are presented as mean \pm SEM. Groups with different superscript letters are significantly different at $P < 0.05$, according to the non-parametric test Kruskal-Wallis (for eWAT and iWAT) and the post-hoc ANOVA Tukey's HSD test (for rWAT and iBAT).

4 DISCUSSION

Previous studies have shown that dietary n-3 fatty acids have an anti-inflammatory effect in rodent models of obesity and human obese subjects (Itoh, Suganami *et al.*, 2007; Todoric, Loffler *et al.*, 2006). To elucidate the possible protection of n-3 fatty acids in fish oil on adipose tissue inflammation, we have performed a feeding experiment with initial 30 male C57BL/6J mice given 5 different diets. In addition, we wanted to investigate if the carbohydrate to protein ratio could influence the development of obesity and the inflammatory response.

4.1 ANIMAL MODELS AND DIETS

The C57BL/6J mouse model has been used in many previous obesity studies, because of its ability to develop a syndrome of obesity, along with hyperinsulinemia, hyperglycemia and hypertension when given free access to a high-fat, high-sucrose diet (Klaus, 2005; Surwit, Feinglos *et al.*, 1995). When only given a low-fat chow diet, these mice remain relatively lean and physically normal. The development of metabolic syndrome related traits, due to a high-fat diet in the C57BL/6J mouse closely parallels to the progression in human disease (i.e. diabetes) (Collins, Martin *et al.*, 2004).

In this study, 4 different high-fat diets were used. Two of the diets had fish oil, rich in n-3 fatty acids as a fat source, and two had corn oil, rich in n-6 fatty acids as a fat source. The fish oil is enriched in EPA and DHA, whereas corn oil is a vegetable oil rich in linoleic acid, which is the predominant n-6 PUFA consumed in the Western diets (Simopoulos, 2000; Zhou and Nilsson, 2001). The two fish oil and corn oil diets had also different carbohydrate to protein ratio (table 2.2).

To verify the different amount of n-3 PUFA and n-6 PUFA in the different diets, fatty acid composition analysis were performed (appendix I, table A.1). The amount of EPA (20:5n-3) and DHA (22:6n-3) were considerably higher in the fish oil diets. Likewise, a notable high amount of LA (18:2n-6) was found in the corn oil diets.

4.2 THE EFFECT OF THE DIFFERENT DIETS ON BODY WEIGHT- AND OBESITY DEVELOPMENT

4.2.1 *Body weight development, adipose tissue weights and histology*

A previous study by Madsen *et al.* (2008) showed that n-6 PUFA were pro-adipogenic when combined with a high-sucrose diet, and non-adipogenic when combined with a high-protein diet (Madsen, Pedersen *et al.*, 2008). These findings were verified in this study. A surprising factor was, however, that the mice receiving fish oil gained more weight than the mice receiving corn oil. We expected that the mice fed fish oil would have gained less weight than the mice fed corn oil, as a previous study by Ruzickova *et al.* (2004) showed that diets rich in n-3 PUFAs from marine origin, EPA and DHA are able to limit the development of obesity in mice. However, the carbohydrate to protein ratio is probably of great importance. In the study by Ruzickova *et al.*, the n-3 PUFA were given in combination with 35.4 % carbohydrate (sucrose) and 20.5 % protein (casein) (Ruzickova, Rossmeisl *et al.*, 2004). In our study the n-3 were given in combination with either 9 % sucrose and 54 % casein, or 43 % sucrose and 20 % casein, in the fish oil + protein diet and fish oil + sucrose diet respectively (table 2.1). The results show that the weight gain was far more pronounced when fish oil was given in combination with sucrose than with protein (figure 3.19). The mice fed fish oil + sucrose also used less energy compared to the ones receiving protein, since they gained over twice as much weight during the feeding experiment. This indicates that the intake of protein enriched diet increased the energy expenditure, i.e. gluconeogenesis and urea synthesis (Madsen, Pedersen *et al.*, 2008). Regarding energy expenditure, the relative expression levels of UCP-1 was observed to be highest in iBAT, as expected (figure 3.26). However, with the exception of rWAT, UCP-1 expression was not significantly different between the groups. A study by Kopecky *et al.* (1996) suggested that high levels of UCP-1 in adipose tissue protects against obesity (Kopecky, Rossmeisl *et al.*, 1996), but there is also reported by Enerback *et al.* (1997), that mice lacking the UCP-1 gene were only cold-sensitive and not developing obesity under normal laboratory conditions (Enerback, Jacobsson *et al.*, 1997). The energy expenditure required to maintain a body temperature is lowest in a thermoneutral environment at 30 °C for mice (Lodhi and Semenkovich, 2009). The mice in our experiment were kept at 22-23 °C during the feeding experiment, so the obesity development might have been affected by this chronic thermal stress condition.

The finding that the mice receiving fish oil + protein had lower adipose tissue weights of eWAT, iWAT, rWAT, iBAT compared to all other 4 groups (figure 3.2, 3.3, 3.4, 3.5), demonstrated that fish oil was not adipogenic in itself. Furthermore, histology pictures of the paraffin- embedded sections of eWAT and iWAT showed smaller adipocytes size for the mice receiving fish oil + protein also compared to the ones receiving the low energy diet (figure 3.12 and 3.13). The anti-adipogenic effect of EPA and DHA has indeed been suggested to reduce accumulation of body fat by limiting both hypertrophy and hyperplasia of adipocytes (Ruzickova, Rossmeisl *et al.*, 2004). Our results demonstrate that this effect is abolished when fish oil is fed in combination with sucrose. Thus, the results clearly suggest that the carbohydrate to protein ratio determines the obesigenic effect of both corn oil and fish oil.

4.2.2 Gene expression levels - Adipocyte differentiation

Obesity may develop as a result of increased adipocyte size due to fat storage (adipocyte hypertrophy) or formation of new adipocytes from precursor cells (adipocyte hyperplasia), or even both (Kubota, Terauchi *et al.*, 1999). Histology pictures from our study showed hypertrophied adipocytes for the mice receiving sucrose in the diet, for both corn oil and fish oil (figure 3.12 and 3.13). Hyperplasia is a result of adipocyte differentiation, thus to get any indication whether new adipocytes were formed in mice fed sucrose, the relative expression levels involved in adipocyte differentiation was measured. We chose PPAR- γ , which has been indicated to play a critical role in the determinant of body fat distribution in mice and humans along with adipogenesis (Tsai and Maeda, 2005). Jones *et al.* (2005) showed that PPAR- γ knockout mice had diminished weight gain when fed a high-fat diet. They also observed that the control mice had higher total body weight and significantly higher expression of PPAR- γ , compared to the PPAR- γ knockout mice (Jones, Barrick *et al.*, 2005). There were also reported that PPAR- γ was higher expressed in adipose tissue in mice fed corn oil in combination with sucrose than in combination with protein (Madsen, Pedersen *et al.*, 2008).

Considering PPAR- γ 's role in adipocyte differentiation, eWAT weights showed that the mice fed sucrose in the diet had the most fat (figure 3.2). Thus, it was expected higher relative gene expression levels of PPAR- γ for the group fed fish oil + sucrose in the diet compared

to the one fed corn oil + protein. However, this study demonstrated that the mice fed fish oil had lower relative expression levels of PPAR- γ in eWAT compared to the mice fed corn oil, when combined both with protein and sucrose (figure 3.8). The statistical difference was only obtained between the fish oil + protein group and corn oil + protein group. The reason for different results compared to the observations by Madsen *et al.* (2008) is not clear, but in the study by Madsen *et al.* the mice were pair fed. This indicates that the relative expression level and activity of PPAR- γ do not necessary have a connection.

Preadipocyte factor-1 plays an important role in inhibition of adipocyte differentiation (Barbu, Hedlund *et al.*, 2009). Villena *et al.* (2008) showed that the mice overexpressing this gene were resistant to high-fat diet induced obesity. The adipose tissue mass was also remarkable reduced, and the mice were highly insulin resistant mainly due to a reduction in insulin stimulated glucose uptake in muscle and adipose tissue (Villena, Choi *et al.*, 2008). In our study the only significant difference of Pref-1 relative expression levels were reached in iBAT, however almost the same expression tendency can be seen in eWAT (figure 3.9). Both groups receiving the protein enriched diets had higher expression levels of Pref-1 than the ones receiving sucrose enriched diets. Moreover, our results showed that the fish oil + sucrose group had the lowest expression of Pref-1 in eWAT, which is reflected on the high eWAT mass (figure 3.2). Likewise, the expression of Pref-1 from the two protein groups were notably higher compared to the sucrose groups in both eWAT and iBAT, which could also be reflected on the lower eWAT and iBAT mass (figure 3.2 and 3.5 respectively). The mice from the protein groups did however not show significantly higher plasma insulin levels, and hence were they possibly not insulin resistant. In this respect it should be noted that insulin levels were measured in plasma obtained from fed- and not fasted mice. Furthermore, the results showed no correlation between the adipose tissue weights (eWAT) for the corn oil + sucrose group who had relatively the same expression level of Pref-1 in eWAT compared to the fish oil + protein group. The adipose tissue weights were overall twice as much for the corn oil + sucrose group compared to the fish oil + protein group.

Plasminogen activator inhibitor-1 is another factor related to obesity. Shimomura *et al.*(1996) observed an increased PAI-1 expression level in visceral fat in rodent model during the development of obesity (Shimomura, Funahashi *et al.*, 1996). Our results showed only

significant difference of PAI-1 expression levels in iWAT, which is a subcutaneous adipose tissue. The highest expression levels were however found in eWAT, where the corn oil + protein group had the highest expression of PAI-1 compared to the other treatment groups. Although the mice in this group were not the most obese, can also other factors such as the levels of triacylglycerol (TAG) and free fatty acids (FFA) be able to stimulate the expression of PAI-1 in adipocytes (Loskutoff and Samad, 1998). Plasma levels of TAG and FFA were not significantly different between the groups, but the corn oil + protein group did not have the lowest levels of the parameters either.

Adiponectin is thought to enhance insulin sensitivity by promoting adipocyte differentiation. A low expression level of adiponectin is observed in obesity and inflammatory states, whereas a high level of adiponectin associated with an accelerated adipocyte differentiation (Fu, Luo *et al.*, 2005). In addition, previous studies have demonstrated that diets enriched in n-3 PUFAs are able to increase the expression level of adiponectin in adipose tissue in rodent models (Flachs, Mohamed-Ali *et al.*, 2006; Neschen, Morino *et al.*, 2006; Todoric, Loffler *et al.*, 2006). Even though there were no significant differences in adiponectin expression levels between the two fish oil groups in any of the adipose tissues, graphically assessments showed a higher expression level for the fish oil + protein group compared to the fish oil + sucrose group in both eWAT and iBAT (figure 3.10). These data suggest that the fish oil in combination with protein may have enhanced the level of adiponectin expression in adipose tissue. Adiponectin might also be able to inhibit adhesion of macrophages into endothelial cells. It is plausible that the lower expression level of adiponectin from the fish oil + sucrose group in eWAT may have contributed to an increased macrophage infiltration in adipose tissue (Xu, Barnes *et al.*, 2003), which can be seen from elevated expression levels of the macrophage markers CD68 and F4/80 for the same group (see section 1.4.1).

The secretion of adiponectin is also reported to be inversely related to the expression levels of proinflammatory adipokines. Gustafson *et al.* (2003) showed that both TNF- α and IL-6 suppress the mRNA expression levels and secretion of adiponectin (Gustafson, Jack *et al.*, 2003). The highest relative expression levels of TNF- α and IL-6 is from the fish oil + sucrose group in eWAT, that is correlated with the lowest expression level of adiponectin for

the same group in eWAT. The expression levels of TNF- α and IL-6 are increased in obese subjects, and the suppression mechanism may result in the lower expression level of adiponectin observed in the fish oil + sucrose group, with the most obese mice. Furthermore, Ajuwon *et al.* (2005) reported that adiponectin is able to inhibit LPS-induced NF- κ B activation, and subsequently decrease the level of IL-6 and increase PPAR- γ (Ajuwon and Spurlock, 2005a)

In conclusion, we presume that the increased adipose tissue mass is due to the sucrose enrichment in the diets, which led to hypertrophied adipocytes. Whether hyperplasia also occurs is not certain based on these results. There are no clear indications from gene expression analysis, thus we need to count cells in order to figure this out.

4.3 THE EFFECT OF THE DIFFERENT DIETS ON ADIPOSE TISSUE INFLAMMATION

4.3.1 Gene expression levels - Macrophage markers, cytokines and chemokines

A major objective in this study was to investigate if n-3 fatty acids in fish oil can protect against adipose tissue inflammation. Obesity is considered as a chronic low-grade inflammatory condition, and hence linked to the infiltration of macrophages in adipose tissue. We could not register any CLS in the histology sections, but the formation of CLS occur late, usually when the cells are dying, thus inflammation can occur even without seeing any CLS (Cinti, Mitchell *et al.*, 2005). The infiltrated macrophages enhance the inflammatory status by secreting several proinflammatory molecules, such as TNF- α and IL-6 (Rausch, Weisberg *et al.*, 2008; Stienstra, Duval *et al.*, 2008). The expression levels from the macrophage-specific markers CD68 and F4/80 can give an indication of the macrophage infiltration status, and thus the degree of adipose tissue inflammation.

The relative expression levels of F4/80 and CD68 indicates that sucrose in the diet had led to infiltration of macrophages, especially in eWAT where significant differences were reached (figure 3.14 and 3.15 respectively). There is a clear pattern showing that the relative expression levels of both F4/80 and CD68 from the fish oil + sucrose group are remarkable higher in all the white adipose tissues compared to the other treatment groups. Numerous of

studies have reported that the consumption of n-3 PUFAs have a favourable effect on adipose tissue inflammation. One of the studies carried out by Todoric *et al.* (2006), presented evidence that the enrichment of n-3 PUFA in a high-fat diet is able to prevent the development of an inflammatory gene expression profile, and macrophage infiltration in the adipose tissue of obese mice. The attenuating effect of n-3 PUFAs on the macrophage surface marker CD68 was highly noticeable (Todoric, Loffler *et al.*, 2006). Our results suggest that the n-3 PUFAs from the fish oil groups more effectively prevent macrophage infiltration when combined with protein rather than sucrose. Xu *et al.* (2003) reported that the macrophage-markers including CD68 and F4/80 were remarkable upregulated in WAT in mice of genetic and high-fat diet induced obesity (Xu, Barnes *et al.*, 2003). The significant higher eWAT mass (g) for the fish oil + sucrose group compared to the groups fed protein might also be reflected on a higher expression of the macrophage-specific genes. The higher relative expression level of MCP-1 in all the adipose tissues (eWAT, iWAT, rWAT and iBAT) for the fish oil +sucrose group, also affect the expression levels of CD68 and F4/80 because of MCP-1 ability to recruit macrophages into adipose tissue (Gustafson, Hammarstedt *et al.*, 2007).

In conclusion, the findings showed no CLS which is a general phenomenon in WAT indicating an inflammatory state. The macrophage markers showed increased expression levels for the mice fed high-sucrose diets. Surprisingly, were the most pronounced expression shown when sucrose was given in combination with fish oil.

CYTOKINES AND CHEMOKINES

The development of inflammation in adipose tissue leads to an increased expression of proinflammatory cytokines and chemokines. Our results also showed that TNF- α , IL-6 and MCP-1 were significantly higher in WAT when mice were fed sucrose than protein, and surprisingly higher when fed fish oil compared to corn oil. The upregulation of these genes in adipose tissue is known to correlate with adiposity and insulin resistance (Dahlman, Kaaman *et al.*, 2005; Lumeng, Deyoung *et al.*, 2007). Previous studies by Hotamisligil *et al.* (1995, 1993) have shown an increased expression level of TNF- α mRNA in adipose tissue from both obese humans and different rodent models of obesity (Hotamisligil, Arner *et al.*, 1995; Hotamisligil, Shargill *et al.*, 1993). TNF- α secreted by adipose tissue macrophages is

considered to be one of the most important mediators of inflammation (Gustafson, Hammarstedt *et al.*, 2007). Lacasa *et al.* (2007) showed that TNF- α stimulated preadipocytes had an increased activation of the NF- κ B pathway consistent with upregulated expression level of IL-6, IL-8, MCP-1 and IL1 β (Lacasa, Taleb *et al.*, 2007). It has also been observed increased mRNA levels and secretion of MCP-1 in human obesity, where the MCP-1 level was doubled compared to nonobese subjects (Dahlman, Kaaman *et al.*, 2005). Skurk *et al.* (2007) also suggested that adipocyte size may be a key determinant of adipokine secretion. There was a notable increased expression of proinflammatory adipokines mainly as a result of hypertrophic adipocytes (Skurk, Alberti-Huber *et al.*, 2007).

We expected that fish oil would reduce inflammation, but still the results seen can indicate the opposite. What distinguishes our study from the previous studies (Ruzickova, Rossmeis *et al.*, 2004; Sierra, Lara-Villoslada *et al.*, 2006), that show an anti-inflammatory effect when using fish oil, is the different amount of sucrose prepared in the diet. Actually, sucrose appears to be a main contributor to adipose tissue inflammation. Earlier studies support this view, among others Morohoshi *et al.* (1995, 1996) demonstrated that isolated human monocyte from healthy volunteers incubated with glucose, gave significantly increased TNF- α and IL-6 mRNA levels when increasing the level of glucose (Morohoshi, Fujisawa *et al.*, 1995; Morohoshi, Fujisawa *et al.*, 1996). Also in a vivo study by Aljada *et al.* (2006) observed that oral glucose ingestion in healthy human subjects resulted in an increased NF- κ B binding and TNF- α expression level (Aljada, Friedman *et al.*, 2006). In addition, increased IL-6 levels have been observed during hyperglycaemic, but not during normoclycemic clamp (Krogh-Madsen, Moller *et al.*, 2004). However, it should be noted that IL-6 and TNF- α levels have been reported to be decreased after a carbohydrate-enriched meal in type 2 diabetes mellitus patients (Manning, Sutherland *et al.*, 2004).

A diet enriched in sucrose also leads to secretion of insulin. Unfortunately, we were not able to obtain statistical difference in this experiment when measuring plasma insulin in the state due to large individual variations within the groups (figure 3.24). However, given that insulin is secreted from the pancreatic β -cells in response to glucose, it should be mentioned that insulin is reported to activate NF- κ B in different cell systems, by inducing phosphorylation of I κ B (Bertrand, Atfi *et al.*, 1998; Bertrand, Desbois-Mouthon *et al.*, 1999; Bertrand, Philippe

et al., 1995; Pandey, He *et al.*, 2002; Zhou and Kuo, 1997). This might be of importance as activation of the NF- κ B system appears to represent a link between obesity, inflammation of adipose tissue, and insulin resistance (Arkan, Hevener *et al.*, 2005; Kim, Kim *et al.*, 2001; Shoelson, Lee *et al.*, 2003). Thus, the glucose component in the diet might contribute to development of adipose tissue inflammation directly, or indirectly by stimulating insulin secretion and thereby NF- κ B activation. Why fish oil fails to reduce inflammation when combined with sucrose remains an open question.

4.4 THE EFFECT OF THE DIFFERENT DIETS ON METABOLISM

In an attempt to gain insight into what metabolic pathways that were influenced by the different diets given, we measured plasma levels of TAG. The lowering effect of fish oil on plasma TAG concentration is well documented (Flachs, Mohamed-Ali *et al.*, 2006; Nascimento, Barbosa-da-Silva *et al.*, 2009; Riediger, Othman *et al.*, 2008). Flachs *et al.* (2006) showed that mice with free access to food enriched with EPA and DHA had lower plasma TAG levels. Our results showed no significant difference between the treatment groups of plasma TAG levels. Again, it should be mentioned that we had plasma obtained from mice in the fed state only. The lack of statistical difference is probably due to large individual variations within the groups. The plasma levels of TAG, indeed tended to be lower in the fish oil + protein group. It was also observed that when fish oil was given in combination with sucrose, the TAG lowering effect was somewhat abolished (figure 3.22). Thus, the TAG lowering effect appears to be more efficient when fish oil is fed in combination with protein.

Another plasma parameter measured was hydroxybutyrate, which was also the only parameter showing significant difference between the treatment groups. Higher hydroxybutyrate indicate higher fatty acids oxidation (Cahill, 2006). Since mice consuming fat combined with protein accumulate less fat in adipose tissue, we can assume that these mice burn fat, whereas mice consuming fat in combination with sucrose store this fat in adipose tissue. If this is true, differences in fatty acid composition should be noticed in adipose tissue. Our results showed that the accumulation of n-3 fatty acids were higher in adipose tissue (eWAT) for the group when fish oil was in combination with sucrose than

protein (table 3.5). Likewise are the results for the corn oil diets, which showed a higher incorporation of n-6 fatty acids in eWAT for the group fed corn oil in combination with sucrose. This is especially pronounced when data is calculated as mg/g sample (absolute values), but can also be seen when data is presented as relative fatty acid composition (table 3.6). This effect was however far less pronounced in RBC. Looking at absolute values in table 3.1, the levels of n-3 are 9.72 % higher in RBC isolated from mice fed fish oil + sucrose than fish oil + protein, whereas the levels of n-3 are 31.95 % higher in eWAT from mice fed fish oil + sucrose than fish oil + protein. The fatty acid composition in RBC has been reported to reflect the amount of fish oil ingested from the diet. Cao *et al.* (2006) showed that fish oil supplements increased the erythrocyte membrane EPA and DHA by 300% (Cao, Schwichtenberg *et al.*, 2006). On the protein based diet we observed 554.55 % higher levels of n-3 in RBC when corn oil was exchanged with fish oil (table 3.1). On the sucrose based diet the difference was 276.19% (table 3.1). Thus, the levels of n-3 fatty acids in RBC were far higher for the fish oil groups compared to the corn oil groups (table 3.1 and 3.2). Although the statistical analysis did not show any significant difference of n-3/n-6 in RBC between the control group and the fish oil + sucrose group, we can see that the incorporation level is quite different. The reason for not obtaining statistical difference is because the data failed the homogeneity of variance test, due to large individual variations within the group. Thus, a non-parametric test; Kruskal-Wallis was used.

Another phenomenon observed from the fatty acid composition results in liver and eWAT (table 3.5 and 3.6), was that sucrose in the diet led to higher amounts of monounsaturated fatty acids than the groups receiving protein. This might be due to the *de novo* fatty acid synthesis, which is increased when fed a high-carbohydrate diet (Chong, Fielding *et al.*, 2007). Stearoyl-CoA desaturase (SCD) is an enzyme catalyzing the synthesis of monounsaturated fatty acids from saturated fatty acids (Flowers and Ntambi, 2009; Ntambi, Miyazaki *et al.*, 2002). The relative expression level of SCD1 was also increased for the mice fed sucrose in the diet, in combination with corn oil and also for the control group (figure 3.25). This might be of importance also in relation to obesity development, as hepatic SCD1 deficiency is reported to be sufficient to protect mice against high-carbohydrate-induced obesity (Miyazaki, Flowers *et al.*, 2007).

The sterol regulatory element-binding proteins (SREBPs) enhance transcription of genes encoding enzymes of cholesterol and lipid synthesis and uptake (Shimomura, Bashmakov *et al.*, 1999). The relative expression level of SREBP-1c was significantly lower for the fish oil + protein compared to the control group (figure 3.25). PUFAs are in general known to reduce expression of SREBP-1c, but the results showed that sucrose in the diet prevented this inhibition.

In conclusion, the results indicate that sucrose may be the main adipogenic factor, because of its ability to direct fat on to adipose tissue, and the fish oil could not prevent this. It also seems like sucrose is attenuating the known positive effects of fish oil, (i.e. TAG lowering effect and SREBP-inhibition).

4.5 FUTURE REMARKS

This study shows that the carbohydrate to protein ratio determines the adipogenic potential of both corn oil and fish oil. Sugar leads to inflammation of adipose tissue. In sharp contrast to our hypothesis, fish oil did not protect the mice against adipose tissue inflammation, but rather enhanced the process compared to corn oil.

Increased levels of cytokines, glucose, insulin, free fatty acids and triacylglycerol indicate insulin resistance. In this study we only have plasma samples from fed-state, so we can not draw any conclusions about the diabetic state. Blood sampling of mice in fasted state should be carried out. In addition, a glucose tolerance test should be performed, which is easier than performing the gold standard hyperinsulinemic euglycemic clamp experiments.

Even though there were not observed any “crown-like structures” in the adipose tissue sections, inflammation can not be excluded. Immunohistochemistry analysis to further assess the macrophage infiltration state with CD68 or F4/80 anti body stain should be performed. These genes are macrophage specific-markers that can detect macrophages in the adipose tissue, thus immunohistochemistry analysis can give a good additional assessment regarding the inflammation state.

The data obtained from this study opens some important questions; are fish oil always healthy? Does sugar also attenuate other reported beneficial effects of fish oil? Even though statistical significance was not obtained, the data indicated that sugar also attenuated the triacylglycerol lowering effect of fish oil. Sucrose was combined with fish oil in this study. Although people are not expected to use sugar on their fish, it is often eaten with other types of carbohydrate rich-food, such as bread and potatoes. Thus, further investigation is needed to elucidate if the question raised also concerns human nutritional recommendations.

5 CONCLUSIONS

The aim for this study was to find out whether the type of dietary fat can modulate inflammation. In addition, was the objective to investigate the diets' interaction of macronutrients in the development of obesity, and estimate whether the carbohydrate to protein ratio could influence the inflammatory response.

What are the effects of the different diets on body weight- and obesity development?

A high carbohydrate to protein ratio in the diet gave the greatest body weight development, and the weight gain was highest in combination with fish oil. Increased adipose tissue mass and hypertrophied adipocytes from histology assessments might be due to the sucrose in the diet as well. Mice fed fish oil + protein had smaller adipocytes compared to the mice fed low energy.

What are the effects of the different diets on adipose tissue inflammation?

A high carbohydrate to protein ratio in the diet led to higher relative expression levels of macrophage markers and proinflammatory cytokines in adipose tissue. Fish oil in the diet did not protect against adipose tissue inflammation when fed in combination with sucrose, compared to corn oil. However, fish oil given in combination with protein tended to lower the inflammatory response. It is also plausible that the glucose or insulin level is responsible for the inflammation process seen in adipose tissue.

What are the effects of the different diets on metabolism?

The TAG lowering effect of fish oil was somewhat abolished when given in combination with sucrose. Sucrose in the diet led to higher accumulation of n-3 fatty acids in combination with fish oil, and higher accumulation of n-6 fatty acids in combination with corn oil in adipose tissue (eWAT). This indicates that sucrose may be the main adipogenic factor. Sucrose in the diet also led to higher amounts of monounsaturated fatty acids in liver and eWAT.

All the findings showed that negative health effects due to a high-fat diet are not dependent on the fat source alone, but also the interaction between the different macronutrients is of significance.

6 REFERENCES

- Agilent Technologies. (2003) RNA 6000 Nano Assay Quick Reference Guide, Available from; http://www.biology.ualberta.ca/facilities/microarray/uploads/documents/Bioanalyzer/G2938-90030_QuickReference.pdf [Access date: 26. November 2008]
- Ajuwon KM, Spurlock ME. (2005a) Adiponectin inhibits LPS-induced NF-kappaB activation and IL-6 production and increases PPARgamma2 expression in adipocytes. *Am J Physiol Regul Integr Comp Physiol* **288**, R1220-5.
- Ajuwon KM, Spurlock ME. (2005b) Palmitate activates the NF-kappaB transcription factor and induces IL-6 and TNFalpha expression in 3T3-L1 adipocytes. *J Nutr* **135**, 1841-6.
- Aljada A, Friedman J, Ghanim H, Mohanty P, Hofmeyer D, Chaudhuri A, Dandona P. (2006) Glucose ingestion induces an increase in intranuclear nuclear factor kappaB, a fall in cellular inhibitor kappaB, and an increase in tumor necrosis factor alpha messenger RNA by mononuclear cells in healthy human subjects. *Metabolism* **55**, 1177-85.
- Arkan MC, Hevener AL, *et al.* (2005) IKK-beta links inflammation to obesity-induced insulin resistance. *Nat Med* **11**, 191-8.
- Astrup A, Meinert Larsen T, Harper A. (2004) Atkins and other low-carbohydrate diets: hoax or an effective tool for weight loss? *Lancet* **364**, 897-9.
- Ayubi M. (2007a) RNA quality control using the Agilent's 2100 Bioanalyzer, Available from; http://biomedicalgenomics.org/RNA_quality_control_Bioanalyzer.html [Access date: 14. March 2009]
- Ayubi M. (2007b) RNA quality control using the NanoDrop ND-1000, Available from; http://biomedicalgenomics.org/RNA_quality_control.html [Access date: 14. March 2009]
- Bagga D, Wang L, Farias-Eisner R, Glaspy JA, Reddy ST. (2003) Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. *Proc Natl Acad Sci U S A* **100**, 1751-6.
- Barbu A, Hedlund GP, Lind J, Carlsson C. (2009) Pref-1 and adipokine expression in adipose tissues of GK and Zucker rats. *Mol Cell Endocrinol* **299**, 163-71.
- Bastard JP, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, Vidal H, Hainque B. (2000) Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *J Clin Endocrinol Metab* **85**, 3338-42.
- Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, Capeau J, Feve B. (2006) Recent advances in the relationship between obesity, inflammation, and insulin resistance. *Eur Cytokine Netw* **17**, 4-12.
- Bastard JP, Maachi M, Van Nhieu JT, Jardel C, Bruckert E, Grimaldi A, Robert JJ, Capeau J, Hainque B. (2002) Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both in vivo and in vitro. *J Clin Endocrinol Metab* **87**, 2084-9.

- Beltowski J. (2003) Adiponectin and resistin--new hormones of white adipose tissue. *Med Sci Monit* **9**, RA55-61.
- Bertrand F, Atfi A, Cadoret A, L'Allemain G, Robin H, Lascols O, Capeau J, Cherqui G. (1998) A role for nuclear factor kappaB in the antiapoptotic function of insulin. *J Biol Chem* **273**, 2931-8.
- Bertrand F, Desbois-Mouthon C, Cadoret A, Prunier C, Robin H, Capeau J, Atfi A, Cherqui G. (1999) Insulin antiapoptotic signaling involves insulin activation of the nuclear factor kappaB-dependent survival genes encoding tumor necrosis factor receptor-associated factor 2 and manganese-superoxide dismutase. *J Biol Chem* **274**, 30596-602.
- Bertrand F, Philippe C, Antoine PJ, Baud L, Groyer A, Capeau J, Cherqui G. (1995) Insulin activates nuclear factor kappa B in mammalian cells through a Raf-1-mediated pathway. *J Biol Chem* **270**, 24435-41.
- Bhattacharya A, Rahman M, Sun D, Fernandes G. (2007) Effect of fish oil on bone mineral density in aging C57BL/6 female mice. *J Nutr Biochem* **18**, 372-9.
- Blok WL, Katan MB, van der Meer JW. (1996) Modulation of inflammation and cytokine production by dietary (n-3) fatty acids. *J Nutr* **126**, 1515-33.
- Blouet C, Mariotti F, Azzout-Marniche D, Bos C, Mathe V, Tome D, Huneau JF. (2006) The reduced energy intake of rats fed a high-protein low-carbohydrate diet explains the lower fat deposition, but macronutrient substitution accounts for the improved glycemic control. *J Nutr* **136**, 1849-54.
- Bluher M. (2008) The inflammatory process of adipose tissue. *Pediatr Endocrinol Rev* **6**, 24-31.
- Cahill GF, Jr. (2006) Fuel metabolism in starvation. *Annu Rev Nutr* **26**, 1-22.
- Calder PC. (2002) Dietary modification of inflammation with lipids. *Proc Nutr Soc* **61**, 345-58.
- Calder PC. (2006) n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nutr* **83**, 1505S-1519S.
- Calder PC, Yaqoob P. (2009) Omega-3 polyunsaturated fatty acids and human health outcomes. *Biofactors*.
- Cancello R, Henegar C, et al. (2005) Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* **54**, 2277-86.
- Cao J, Schwichtenberg KA, Hanson NQ, Tsai MY. (2006) Incorporation and clearance of omega-3 fatty acids in erythrocyte membranes and plasma phospholipids. *Clin Chem* **52**, 2265-72.
- Cecil JE, Watt P, Palmer CN, Hetherington M. (2006) Energy balance and food intake: the role of PPARgamma gene polymorphisms. *Physiol Behav* **88**, 227-33.
- Chen A, Mumick S, et al. (2005) Diet induction of monocyte chemoattractant protein-1 and its impact on obesity. *Obes Res* **13**, 1311-20.

- Chong MF, Fielding BA, Frayn KN. (2007) Metabolic interaction of dietary sugars and plasma lipids with a focus on mechanisms and de novo lipogenesis. *Proc Nutr Soc* **66**, 52-9.
- Cinti S, Mitchell G, *et al.* (2005) Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* **46**, 2347-55.
- Clement K, Viguier N, *et al.* (2004) Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. *FASEB J* **18**, 1657-69.
- Collins S, Martin TL, Surwit RS, Robidoux J. (2004) Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. *Physiol Behav* **81**, 243-8.
- Connor WE, Connor SL. (1997) Should a low-fat, high-carbohydrate diet be recommended for everyone? The case for a low-fat, high-carbohydrate diet. *N Engl J Med* **337**, 562-3; discussion 566-7.
- Cormack DH. (2001) Introduction to Histology. In 'Essential Histology' pp. 2-4. (Lippincott Williams & Wilkins: Philadelphia)
- Cottam DR, Mattar SG, Barinas-Mitchell E, Eid G, Kuller L, Kelley DE, Schauer PR. (2004) The chronic inflammatory hypothesis for the morbidity associated with morbid obesity: implications and effects of weight loss. *Obes Surg* **14**, 589-600.
- Cuschieri J, Bulger E, Grinsell R, Garcia I, Maier RV. (2008) Insulin regulates macrophage activation through activin A. *Shock* **29**, 285-90.
- Dahlman I, Kaaman M, *et al.* (2005) A unique role of monocyte chemoattractant protein 1 among chemokines in adipose tissue of obese subjects. *J Clin Endocrinol Metab* **90**, 5834-40.
- de Castro JM. (1999) Behavioral genetics of food intake regulation in free-living humans. *Nutrition* **15**, 550-4.
- de Ferranti S, Mozaffarian D. (2008) The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences. *Clin Chem* **54**, 945-55.
- De Groote G, De Laporte A, Dhondt G, Christophe A. (2008) Improvement in the plasma omega-3 index by the use of a fish oil-enriched spread. *Ann Nutr Metab* **53**, 23-8.
- de Luca C, Olefsky JM. (2006) Stressed out about obesity and insulin resistance. *Nat Med* **12**, 41-2; discussion 42.
- de Luca C, Olefsky JM. (2008) Inflammation and insulin resistance. *FEBS Lett* **582**, 97-105.
- Diez JJ, Iglesias P. (2003) The role of the novel adipocyte-derived hormone adiponectin in human disease. *Eur J Endocrinol* **148**, 293-300.
- DRG Diagnostics. (2007) Insulin Mouse Ultrasensitive ELISA EIA-3440. Enzyme Immunoassay for the quantitative determination of insulin in mouse serum or plasma. DRG Instruments GmbH Marburg.
- Dvergnes K, Skeie G. (2009) [The development of BMI in 4-year-olds in Tromso 1980 to 2005]. *Tidsskr Nor Laegeforen* **129**, 13-6.

- Enerback S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper ME, Kozak LP. (1997) Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature* **387**, 90-4.
- Fantuzzi G. (2005) Adipose tissue, adipokines, and inflammation. *J Allergy Clin Immunol* **115**, 911-9; quiz 920.
- Flachs P, Mohamed-Ali V, Horakova O, Rossmeisl M, Hosseinzadeh-Attar MJ, Hensler M, Ruzickova J, Kopecky J. (2006) Polyunsaturated fatty acids of marine origin induce adiponectin in mice fed a high-fat diet. *Diabetologia* **49**, 394-7.
- Flegal KM, Carroll MD, Ogden CL, Johnson CL. (2002) Prevalence and trends in obesity among US adults, 1999-2000. *JAMA* **288**, 1723-7.
- Flowers MT, Ntambi JM. (2008) Role of stearoyl-coenzyme A desaturase in regulating lipid metabolism. *Curr Opin Lipidol* **19**, 248-56.
- Flowers MT, Ntambi JM. (2009) Stearoyl-CoA desaturase and its relation to high-carbohydrate diets and obesity. *Biochim Biophys Acta* **1791**, 85-91.
- Fu Y, Luo N, Klein RL, Garvey WT. (2005) Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. *J Lipid Res* **46**, 1369-79.
- Fulgoni VL, 3rd. (2008) Current protein intake in America: analysis of the National Health and Nutrition Examination Survey, 2003-2004. *Am J Clin Nutr* **87**, 1554S-1557S.
- Gil A. (2002) Polyunsaturated fatty acids and inflammatory diseases. *Biomed Pharmacother* **56**, 388-96.
- Giltay EJ, Elbers JM, Gooren LJ, Emeis JJ, Kooistra T, Asscheman H, Stehouwer CD. (1998) Visceral fat accumulation is an important determinant of PAI-1 levels in young, nonobese men and women: modulation by cross-sex hormone administration. *Arterioscler Thromb Vasc Biol* **18**, 1716-22.
- Gustafson B, Hammarstedt A, Andersson CX, Smith U. (2007) Inflamed adipose tissue: a culprit underlying the metabolic syndrome and atherosclerosis. *Arterioscler Thromb Vasc Biol* **27**, 2276-83.
- Gustafson B, Jack MM, Cushman SW, Smith U. (2003) Adiponectin gene activation by thiazolidinediones requires PPAR gamma 2, but not C/EBP alpha-evidence for differential regulation of the aP2 and adiponectin genes. *Biochem Biophys Res Commun* **308**, 933-9.
- Haluzik M, Colombo C, *et al.* (2004) Genetic background (C57BL/6J versus FVB/N) strongly influences the severity of diabetes and insulin resistance in ob/ob mice. *Endocrinology* **145**, 3258-64.
- Handeland M, Hjelmestaeth J. (2009) [Obesity epidemics--we must start with the children]. *Tidsskr Nor Lægeforen* **129**, 12.
- Harizi H, Corcuff JB, Gualde N. (2008) Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. *Trends Mol Med* **14**, 461-9.
- Haslam D, Sattar N, Lean M. (2006) ABC of obesity. Obesity--time to wake up. *BMJ* **333**, 640-2.
- Haslam DW, James WP. (2005) Obesity. *Lancet* **366**, 1197-209.

- Heilbronn LK, Campbell LV. (2008) Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. *Curr Pharm Des* **14**, 1225-30.
- Horton JD. (2002) Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. *Biochem Soc Trans* **30**, 1091-5.
- Hotamisligil GS. (1999) The role of TNF α and TNF receptors in obesity and insulin resistance. *J Intern Med* **245**, 621-5.
- Hotamisligil GS. (2006) Inflammation and metabolic disorders. *Nature* **444**, 860-7.
- Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. (1995) Increased adipose tissue expression of tumor necrosis factor- α in human obesity and insulin resistance. *J Clin Invest* **95**, 2409-15.
- Hotamisligil GS, Shargill NS, Spiegelman BM. (1993) Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science* **259**, 87-91.
- Hudgins LC. (2000) Effect of high-carbohydrate feeding on triglyceride and saturated fatty acid synthesis. *Proc Soc Exp Biol Med* **225**, 178-83.
- Imbeaud S, Graudens E, Boulanger V, Barlet X, Zaborski P, Eveno E, Mueller O, Schroeder A, Auffray C. (2005) Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. *Nucleic Acids Res* **33**, e56.
- Itoh M, Suganami T, *et al.* (2007) Increased adiponectin secretion by highly purified eicosapentaenoic acid in rodent models of obesity and human obese subjects. *Arterioscler Thromb Vasc Biol* **27**, 1918-25.
- Jiang G, Li Z, *et al.* (2005) Prevention of obesity in mice by antisense oligonucleotide inhibitors of stearoyl-CoA desaturase-1. *J Clin Invest* **115**, 1030-8.
- Jones JR, Barrick C, *et al.* (2005) Deletion of PPAR γ in adipose tissues of mice protects against high fat diet-induced obesity and insulin resistance. *Proc Natl Acad Sci U S A* **102**, 6207-12.
- Kadowaki T, Hara K, *et al.* (2002) The role of PPAR γ in high-fat diet-induced obesity and insulin resistance. *J Diabetes Complications* **16**, 41-5.
- Kahn SE, Hull RL, Utzschneider KM. (2006) Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* **444**, 840-6.
- Kanda H, Tateya S, *et al.* (2006) MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* **116**, 1494-505.
- Kelly T, Yang W, Chen CS, Reynolds K, He J. (2008) Global burden of obesity in 2005 and projections to 2030. *Int J Obes (Lond)* **32**, 1431-7.
- Kempf K, Rose B, Herder C, Kleophas U, Martin S, Kolb H. (2006) Inflammation in metabolic syndrome and type 2 diabetes: Impact of dietary glucose. *Ann N Y Acad Sci* **1084**, 30-48.
- Kim JK, Kim YJ, *et al.* (2001) Prevention of fat-induced insulin resistance by salicylate. *J Clin Invest* **108**, 437-46.

- Klaus S. (2005) Increasing the protein:carbohydrate ratio in a high-fat diet delays the development of adiposity and improves glucose homeostasis in mice. *J Nutr* **135**, 1854-8.
- Kopecky J, Rossmeisl M, Hodny Z, Syrový I, Horáková M, Kolarová P. (1996) Reduction of dietary obesity in aP2-Ucp transgenic mice: mechanism and adipose tissue morphology. *Am J Physiol* **270**, E776-86.
- Krogh-Madsen R, Møller K, Dela F, Kronborg G, Jauffred S, Pedersen BK. (2004) Effect of hyperglycemia and hyperinsulinemia on the response of IL-6, TNF- α , and FFAs to low-dose endotoxemia in humans. *Am J Physiol Endocrinol Metab* **286**, E766-72.
- Kubota N, Terauchi Y, *et al.* (1999) PPAR γ mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol Cell* **4**, 597-609.
- Lacasa D, Taleb S, Keophiphath M, Miranville A, Clement K. (2007) Macrophage-secreted factors impair human adipogenesis: involvement of proinflammatory state in preadipocytes. *Endocrinology* **148**, 868-77.
- Lacroix M, Gaudichon C, Martin A, Morens C, Mathe V, Tome D, Huneau JF. (2004) A long-term high-protein diet markedly reduces adipose tissue without major side effects in Wistar male rats. *Am J Physiol Regul Integr Comp Physiol* **287**, R934-42.
- Lara-Castro C, Fu Y, Chung BH, Garvey WT. (2007) Adiponectin and the metabolic syndrome: mechanisms mediating risk for metabolic and cardiovascular disease. *Curr Opin Lipidol* **18**, 263-70.
- Laviola L, Perrini S, Cignarelli A, Giorgino F. (2006) Insulin signalling in human adipose tissue. *Arch Physiol Biochem* **112**, 82-8.
- Lee K, Villena JA, Moon YS, Kim KH, Lee S, Kang C, Sul HS. (2003) Inhibition of adipogenesis and development of glucose intolerance by soluble preadipocyte factor-1 (Pref-1). *J Clin Invest* **111**, 453-61.
- Liang G, Yang J, Horton JD, Hammer RE, Goldstein JL, Brown MS. (2002) Diminished hepatic response to fasting/refeeding and liver X receptor agonists in mice with selective deficiency of sterol regulatory element-binding protein-1c. *J Biol Chem* **277**, 9520-8.
- Liang X, Kanjanabuch T, *et al.* (2006) Plasminogen activator inhibitor-1 modulates adipocyte differentiation. *Am J Physiol Endocrinol Metab* **290**, E103-E113.
- Lin HH, Faunce DE, *et al.* (2005) The macrophage F4/80 receptor is required for the induction of antigen-specific efferent regulatory T cells in peripheral tolerance. *J Exp Med* **201**, 1615-25.
- Lodhi IJ, Semenkovich CF. (2009) Why we should put clothes on mice. *Cell Metab* **9**, 111-2.
- Loskutoff DJ, Samad F. (1998) The adipocyte and hemostatic balance in obesity: studies of PAI-1. *Arterioscler Thromb Vasc Biol* **18**, 1-6.
- Lumeng CN, Deyoung SM, Saltiel AR. (2007) Macrophages block insulin action in adipocytes by altering expression of signaling and glucose transport proteins. *Am J Physiol Endocrinol Metab* **292**, E166-74.
- Ma H, Shieh K-J, Chen G, Qiao XT, Chuang M-Y. (2006) Application of Real-time Polymerase Chain Reaction (RT-PCR). *The Journal of American Science*.

- Madsen L, Pedersen LM, *et al.* (2008) cAMP-dependent signaling regulates the adipogenic effect of n-6 polyunsaturated fatty acids. *J Biol Chem* **283**, 7196-205.
- Malterud K, Tonstad S. (2009) Preventing obesity: Challenges and pitfalls for health promotion. *Patient Educ Couns.*
- Manning PJ, Sutherland WH, Hendry G, de Jong SA, McGrath M, Williams SM. (2004) Changes in circulating postprandial proinflammatory cytokine concentrations in diet-controlled type 2 diabetes and the effect of ingested fat. *Diabetes Care* **27**, 2509-11.
- Matsuzaka T, Shimano H, *et al.* (2002) Cloning and characterization of a mammalian fatty acyl-CoA elongase as a lipogenic enzyme regulated by SREBPs. *J Lipid Res* **43**, 911-20.
- MAXMAT PL. (2003) MAXMAT™ PL Multi-purpose diagnostic analyzer system. User's Manual. MAXMAT S.A. France, Montpellier.
- McGill JB, Schneider DJ, Arfken CL, Lucore CL, Sobel BE. (1994) Factors responsible for impaired fibrinolysis in obese subjects and NIDDM patients. *Diabetes* **43**, 104-9.
- McKnight AJ, Macfarlane AJ, Dri P, Turley L, Willis AC, Gordon S. (1996) Molecular cloning of F4/80, a murine macrophage-restricted cell surface glycoprotein with homology to the G-protein-linked transmembrane 7 hormone receptor family. *J Biol Chem* **271**, 486-9.
- Medzhitov R. (2001) Toll-like receptors and innate immunity. *Nat Rev Immunol* **1**, 135-45.
- Melancon S, Bachelard H, Badeau M, Bourgoin F, Pitre M, Lariviere R, Nadeau A. (2006) Effects of high-sucrose feeding on insulin resistance and hemodynamic responses to insulin in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol* **290**, H2571-81.
- Miyazaki M, Flowers MT, Sampath H, Chu K, Oztzelberger C, Liu X, Ntambi JM. (2007) Hepatic stearoyl-CoA desaturase-1 deficiency protects mice from carbohydrate-induced adiposity and hepatic steatosis. *Cell Metab* **6**, 484-96.
- Moon YS, Smas CM, Lee K, Villena JA, Kim KH, Yun EJ, Sul HS. (2002) Mice lacking paternally expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity. *Mol Cell Biol* **22**, 5585-92.
- Morohoshi M, Fujisawa K, Uchimura I, Numano F. (1995) The effect of glucose and advanced glycosylation end products on IL-6 production by human monocytes. *Ann N Y Acad Sci* **748**, 562-70.
- Morohoshi M, Fujisawa K, Uchimura I, Numano F. (1996) Glucose-dependent interleukin 6 and tumor necrosis factor production by human peripheral blood monocytes in vitro. *Diabetes* **45**, 954-9.
- Mueller O, Lightfoot S, Schroeder A. (2004) RNA Integrity Number (RIN)- Standardization of RNA Quality Control. *Agilent Application Note*, Publication Number-5989-1165EN, 1-8.
- Nascimento FA, Barbosa-da-Silva S, Fernandes-Santos C, Mandarim-de-Lacerda CA, Aguilá MB. (2009) Adipose tissue, liver and pancreas structural alterations in C57BL/6 mice fed high-fat-high-sucrose diet supplemented with fish oil (n-3 fatty acid rich oil). *Exp Toxicol Pathol.*

- Nedergaard J, Golozoubova V, Matthias A, Asadi A, Jacobsson A, Cannon B. (2001) UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency. *Biochim Biophys Acta* **1504**, 82-106.
- Neschen S, Morino K, Rossbacher JC, Pongratz RL, Cline GW, Sono S, Gillum M, Shulman GI. (2006) Fish oil regulates adiponectin secretion by a peroxisome proliferator-activated receptor-gamma-dependent mechanism in mice. *Diabetes* **55**, 924-8.
- NIFES. (2005a) 279-RT Reaction. MET.MOL-64. National institute of nutrition and seafood research, Bergen.
- NIFES. (2005b) 281-RNA Rensing og RNA Kvantitet. MET.MOL-66. Nasjonalt institutt for ernærings- og sjømatforskning, Bergen.
- NIFES. (2009) 041-Fatty acid composition of total lipids by GLC. MET.NÆR.01-02. National institute of nutrition and seafood research, Bergen.
- Ntambi JM, Miyazaki M, *et al.* (2002) Loss of stearyl-CoA desaturase-1 function protects mice against adiposity. *Proc Natl Acad Sci U S A* **99**, 11482-6.
- Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM. (2006) Prevalence of overweight and obesity in the United States, 1999-2004. *JAMA* **295**, 1549-55.
- Panaro NJ, Yuen PK, Sakazume T, Fortina P, Kricka LJ, Wilding P. (2000) Evaluation of DNA fragment sizing and quantification by the agilent 2100 bioanalyzer. *Clin Chem* **46**, 1851-3.
- Pandey SK, He HJ, Chesley A, Juhaszova M, Crow MT, Bernier M. (2002) Wortmannin-sensitive pathway is required for insulin-stimulated phosphorylation of inhibitor kappaBalpha. *Endocrinology* **143**, 375-85.
- Qatanani M, Szwegold NR, Greaves DR, Ahima RS, Lazar MA. (2009) Macrophage-derived human resistin exacerbates adipose tissue inflammation and insulin resistance in mice. *J Clin Invest*.
- Qin Y, Dalen KT, Gustafsson JA, Nebb HI. (2009) Regulation of hepatic fatty acid elongase 5 by LXRAalpha-SREBP-1c. *Biochim Biophys Acta* **1791**, 140-7.
- Rangwala SM, Lazar MA. (2004) Peroxisome proliferator-activated receptor gamma in diabetes and metabolism. *Trends Pharmacol Sci* **25**, 331-6.
- Rausch ME, Weisberg S, Vardhana P, Tortoriello DV. (2008) Obesity in C57BL/6J mice is characterized by adipose tissue hypoxia and cytotoxic T-cell infiltration. *Int J Obes (Lond)* **32**, 451-63.
- Riediger ND, Othman R, Fitz E, Pierce GN, Suh M, Moghadasian MH. (2008) Low n-6:n-3 fatty acid ratio, with fish- or flaxseed oil, in a high fat diet improves plasma lipids and beneficially alters tissue fatty acid composition in mice. *Eur J Nutr* **47**, 153-60.
- Ruzickova J, Rossmeisl M, Prazak T, Flachs P, Sponarova J, Veck M, Tvrzicka E, Bryhn M, Kopecky J. (2004) Omega-3 PUFA of marine origin limit diet-induced obesity in mice by reducing cellularity of adipose tissue. *Lipids* **39**, 1177-85.
- Schaefer EJ, Gleason JA, Dansinger ML. (2009) Dietary fructose and glucose differentially affect lipid and glucose homeostasis. *J Nutr* **139**, 1257S-1262S.

- Schroeder A, Mueller O, *et al.* (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol* **7**, 3.
- Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. (2006) TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest* **116**, 3015-25.
- Shimomura I, Bashmakov Y, Ikemoto S, Horton JD, Brown MS, Goldstein JL. (1999) Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. *Proc Natl Acad Sci U S A* **96**, 13656-61.
- Shimomura I, Funahashi T, *et al.* (1996) Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. *Nat Med* **2**, 800-3.
- Shoelson SE, Herrero L, Naaz A. (2007) Obesity, inflammation, and insulin resistance. *Gastroenterology* **132**, 2169-80.
- Shoelson SE, Lee J, Yuan M. (2003) Inflammation and the IKK beta/I kappa B/NF-kappa B axis in obesity- and diet-induced insulin resistance. *Int J Obes Relat Metab Disord* **27 Suppl 3**, S49-52.
- Sierra S, Lara-Villoslada F, Comalada M, Olivares M, Xaus J. (2006) Dietary fish oil n-3 fatty acids increase regulatory cytokine production and exert anti-inflammatory effects in two murine models of inflammation. *Lipids* **41**, 1115-25.
- Simopoulos AP. (2000) Human requirement for N-3 polyunsaturated fatty acids. *Poult Sci* **79**, 961-70.
- Simopoulos AP. (2001) Evolutionary aspects of diet and essential fatty acids. *World Rev Nutr Diet* **88**, 18-27.
- Simopoulos AP. (2002a) The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother* **56**, 365-79.
- Simopoulos AP. (2002b) Omega-3 fatty acids in inflammation and autoimmune diseases. *J Am Coll Nutr* **21**, 495-505.
- Simopoulos AP. (2003) Importance of the ratio of omega-6/omega-3 essential fatty acids: evolutionary aspects. *World Rev Nutr Diet* **92**, 1-22.
- Skurk T, Alberti-Huber C, Herder C, Hauner H. (2007) Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* **92**, 1023-33.
- Skurk T, Hauner H. (2004) Obesity and impaired fibrinolysis: role of adipose production of plasminogen activator inhibitor-1. *Int J Obes Relat Metab Disord* **28**, 1357-64.
- Skálhegg SB. (2007) Protein. In 'Mat og medisin '. (Eds CA Drevon, G-EA Bjørneboe and R Blomhoff) pp. 118. (HøyskoleForlaget: Kristiansand)
- Smith CJ, Osborn AM. (2009) Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol Ecol* **67**, 6-20.
- Sodowich BI, Fadl I, Burns C. (2007) Method validation of in vitro RNA transcript analysis on the Agilent 2100 Bioanalyzer. *Electrophoresis* **28**, 2368-78.

- Solt LA, May MJ. (2008) The I κ B kinase complex: master regulator of NF- κ B signaling. *Immunol Res* **42**, 3-18.
- Stienstra R, Duval C, Keshtkar S, van der Laak J, Kersten S, Muller M. (2008) Peroxisome proliferator-activated receptor gamma activation promotes infiltration of alternatively activated macrophages into adipose tissue. *J Biol Chem* **283**, 22620-7.
- Stienstra R, Duval C, Muller M, Kersten S. (2007) PPARs, Obesity, and Inflammation. *PPAR Res* **2007**, 95974.
- Sul HS, Smas C, Mei B, Zhou L. (2000) Function of pref-1 as an inhibitor of adipocyte differentiation. *Int J Obes Relat Metab Disord* **24 Suppl 4**, S15-9.
- Surwit RS, Feinglos MN, Rodin J, Sutherland A, Petro AE, Opara EC, Kuhn CM, Rebuffe-Scrive M. (1995) Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. *Metabolism* **44**, 645-51.
- Tatsumi K, Ohashi K, Taminishi S, Okano T, Yoshioka A, Shima M. (2008) Reference gene selection for real-time RT-PCR in regenerating mouse livers. *Biochem Biophys Res Commun* **374**, 106-10.
- Todoric J, Loffler M, Huber J, Bilban M, Reimers M, Kadl A, Zeyda M, Waldhausl W, Stulnig TM. (2006) Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids. *Diabetologia* **49**, 2109-19.
- Tsai YS, Maeda N. (2005) PPARgamma: a critical determinant of body fat distribution in humans and mice. *Trends Cardiovasc Med* **15**, 81-5.
- Uchôa Junqueira LC, Carneiro J. (2005) Histology & Its Methods of Study. In 'Basic histology: text & atlas' pp. 1-2. (McGraw-Hill Professional: New York)
- Ulset E, Undheim R, Malterud K. (2007) [Has the obesity epidemic reached Norway?]. *Tidsskr Nor Lægeforen* **127**, 34-7.
- Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. (1997) Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* **389**, 610-4.
- Valasek MA, Repa JJ. (2005) The power of real-time PCR. *Adv Physiol Educ* **29**, 151-9.
- van den Berg TK, Kraal G. (2005) A function for the macrophage F4/80 molecule in tolerance induction. *Trends Immunol* **26**, 506-9.
- van Schothorst EM, Keijer J, Pennings JL, Opperhuizen A, van den Brom CE, Kohl T, Franssen-van Hal NL, Hoebee B. (2006) Adipose gene expression response of lean and obese mice to short-term dietary restriction. *Obesity (Silver Spring)* **14**, 974-9.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, RESEARCH0034.

- Venkatraman JT, Chu WC. (1999) Effects of dietary omega-3 and omega-6 lipids and vitamin E on serum cytokines, lipid mediators and anti-DNA antibodies in a mouse model for rheumatoid arthritis. *J Am Coll Nutr* **18**, 602-13.
- Villena JA, Choi CS, Wang Y, Kim S, Hwang YJ, Kim YB, Cline G, Shulman GI, Sul HS. (2008) Resistance to high-fat diet-induced obesity but exacerbated insulin resistance in mice overexpressing preadipocyte factor-1 (Pref-1): a new model of partial lipodystrophy. *Diabetes* **57**, 3258-66.
- Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. (2003) Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* **112**, 1796-808.
- Wellen KE, Fucho R, *et al.* (2007) Coordinated regulation of nutrient and inflammatory responses by STAMP2 is essential for metabolic homeostasis. *Cell* **129**, 537-48.
- Westerterp-Plantenga MS, Nieuwenhuizen A, Tome D, Soenen S, Westerterp KR. (2009) Dietary Protein, Weight Loss, and Weight Maintenance. *Annu Rev Nutr*.
- WHO. (2000) Obesity: preventing and managing the global epidemic. Report of a WHO consultation. *World Health Organ Tech Rep Ser* **894**, i-xii, 1-253.
- Wiecha JL, Peterson KE, Ludwig DS, Kim J, Sobol A, Gortmaker SL. (2006) When children eat what they watch: impact of television viewing on dietary intake in youth. *Arch Pediatr Adolesc Med* **160**, 436-42.
- Wu D, Ren Z, Pae M, Guo W, Cui X, Merrill AH, Meydani SN. (2007) Aging up-regulates expression of inflammatory mediators in mouse adipose tissue. *J Immunol* **179**, 4829-39.
- Xu H, Barnes GT, *et al.* (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* **112**, 1821-30.
- Yan J, Burman A, Nichols C, Alila L, Showe LC, Showe MK, Boyer BB, Barnes BM, Marr TG. (2006) Detection of differential gene expression in brown adipose tissue of hibernating arctic ground squirrels with mouse microarrays. *Physiol Genomics* **25**, 346-53.
- Yang J, Craddock L, Hong S, Liu ZM. (2009) AMP-activated protein kinase suppresses LXR-dependent sterol regulatory element-binding protein-1c transcription in rat hepatoma McA-RH7777 cells. *J Cell Biochem* **106**, 414-26.
- Yudkin JS, Kumari M, Humphries SE, Mohamed-Ali V. (2000) Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis* **148**, 209-14.
- Zhou G, Kuo MT. (1997) NF-kappaB-mediated induction of mdr1b expression by insulin in rat hepatoma cells. *J Biol Chem* **272**, 15174-83.
- Zhou L, Nilsson A. (2001) Sources of eicosanoid precursor fatty acid pools in tissues. *J Lipid Res* **42**, 1521-42.



APPENDIX

Appendix I -Fatty acid composition

Table A.1: Fatty acid composition of the diets expressed in mg fatty acid/g sample

Fatty acids mg/fatty acid/g sample	Diets				
	Low energy	Fish oil + Protein	Fish oil + Sucrose	Corn oil + Protein	Corn oil + Sucrose
SFA					
14:0	0,15	9,7	9,9	0,3	0,3
15:0	0,00	0,8	0,8	0,0	0,0
16:0	6,95	30,3	31,0	24,0	24,0
17:0	0,07	0,6	0,7	0,0	0,0
18:0	2,13	5,9	6,8	4,0	4,1
20:0	0,17	0,8	1,1	0,0	0,0
22:0	0,00	0,0	0,0	0,0	0,0
24:0	0,10	0,0	0,0	0,1	0,0
MUFA					
14:1n-9	0,00	0,0	0,0	0,0	0,0
16:1n-9	0,00	0,7	0,6	0,0	0,0
16:1n-7	0,09	11,2	11,3	0,4	0,4
18:1n-11	0,00	0,0	0,0	0,0	0,0
18:1n-9	15,72	29,8	29,4	60,9	60,9
18:1n-7	1,08	4,5	5,3	1,3	1,3
20:1n-11	0,00	0,1	0,0	0,0	0,0
20:1n-9	0,15	2,2	2,3	0,3	0,3
20:1n-7	0,00	0,3	0,3	0,0	0,0
22:1n-11	0,00	1,6	1,5	0,0	0,0
22:1n-9	0,00	0,0	0,0	0,0	0,0
24:1n-9	0,00	0,6	0,6	0,0	0,0
n-6					
18:2n-6	32,48	37,3	39,0	117,8	117,3
20:2n-6	0,00	0,0	0,0	0,0	0,0
20:3n-6	0,00	0,0	0,0	0,0	0,0
20:4n-6	0,22	1,6	1,6	0,0	0,0
n-3					
16:3n-3	0,00	1,5	1,5	0,0	0,0
16:4n-3	0,15	3,4	3,2	1,2	1,1
18:3n-3	3,39	1,8	5,1	1,6	1,6
18:4n-3	0,00	5,2	5,3	0,0	0,0
20:4n-3	0,00	1,5	0,7	0,0	0,0
20:5n-3	0,00	26,5	26,7	0,4	0,5
22:5n-3	0,00	2,8	2,8	0,0	0,0
22:6n-3	0,00	17,9	18,0	0,2	0,3
\sum n-3	3,54	60,6	63,4	3,3	3,5
\sum n-6	32,70	38,9	40,7	117,8	117,3
n-3/n-6	0,1	1,6	1,6	0,0	0,0

SFA: saturated fatty acid, MUFA: monounsaturated fatty acid

Chemicals and reagents

Appendix II- RNA extraction

Table A.2: Chemicals and reagents used in RNA extraction.

Product name	Vendor
Trizol	Invitrogen art. no. 15596-026, USA
Chloroform	Merck, Germany
Isopropanol (2-propanol)	Arcus kjemi, Norway
Ethanol	Arcus kjemi, Norway
DEPC	Sigma art. no. F32490, USA
RNase free ddH ₂ O	MilliQ Gradient, Lab-tec, Norway
DNA free kit	Ambion art. no. 1906, USA

Appendix III-RNA quality on Bioanalyzer (RNA 6000 Nano)

Table A.3: Chemicals and reagents used in RNA quality on Bioanalyzer.

Product name	Vendor
RNA 6000 Nano LabChip kit	Agilent Technologies art. nr 5065-4476
RNA 6000 ladder	Ambion art. nr 7152
RNase free ddH ₂ O	MilliQ Gradient, Lab-tec, Norway

Appendix IV-RT-reaction

Table A.4: Chemicals and reagents used in RT-reaction.

Product name	Vendor
TaqMan RT buffer 10X	Applied Biosystems art. no. N808 0234
25 mM Magnesium chlorid	Applied Biosystems art. no. N808 0234
10 mM deoxyNTPs	Applied Biosystems art. no. N808 0234
50 µM Oligo d(T)16 primer	Applied Biosystems art. no. N808 0234
RNase inhibitor (20 U/µl)	Applied Biosystems art. no. N808 0234
Multiscribe reverse transcriptase (50 U/µl)	Applied Biosystems art. no. N808 0234
RNase free ddH ₂ O	MilliQ Gradient, Lab-tec, Norway

Appendix V- Quantitative Real-Time PCR

Table A.5: Chemicals and reagents used in quantitative real- time PCR.

Product name	Vendor
SYBR GREEN Master	Roche, Norway
Primer (see table A.6)	Invitrogen Ltd, UK
RNase free ddH ₂ O	MilliQ Gradient, Lab-tec, Norway

Table A.6: List of primers obtained from Invitrogen Ltd, UK used in quantitative real- time PCR.

Housekeeping gene	Sequence 5' → 3'
TBP	Forward ACC CTT CAC CAA TGA CTC CTA TG
	Reverse ATG ATG ACT GCA GCA AAT CGC
Calnexin	Forward GCA GCG ACC TAT GAT TGA CAA CC
	Reverse GCT CCA AAC CAA TAG CAC TGA AAG G
Beta-actin	Forward ATG GGT CAG AAG GAC TCC TAC G
	Reverse AGT GGT ACG ACC AGA GGC ATA C
Primer	Sequence 5' → 3'
TNF- alpha	Forward CCC TCA CAC TCA GAT CAT CTT
	Reverse GCT ACG ACG TGG GCT ACA G
IL-6 N	Forward CCT CTC TGC AAG AGA CTT CC
	Reverse AGA ATT GCC ATT GCA CAA CTC
MCP-1	Forward GTG TTG GCT CAG CCA GAT GC
	Reverse GCT TGG TGA CAA AAA CTA
PAI1	Forward AGC GGG ACC TAG AGC TGG TC
	Reverse CCA GTA AGT CAT TGA TCA TAC CTT TGGT
STAMP2	Forward TCA AAT GCG GAA TAC CTT GCT
	Reverse GCA TCT AGT GTT CCT GAC TGG A
F4/80	Forward CTT TGG CTA TGG GCT CTC AGT C
	Reverse GCA AGG AGG ACA GAG TTT ATC GTG
CD68	Forward CTT CCC AGA GGC AGC ACA G
	Reverse AAT GAT GAG AGG CAG CAA GAG G
PPAR-gamma	Forward ACA GCA AAT CTC TGT TTT ATG C
	Reverse TGC TGG AGA AAT CAA CTG TGG
Pref-1	Forward TTC TGC GAA ATA GAC GTT CGG
	Reverse TCG TTC TCG CAT GGG TTA GG
Adiponectin	Forward GAT GGC AGA GAT GGC ACT CC
	Reverse CTT GCC AGT GCT GCC GTC AT
UCP1	Forward AGC CGG CTT AAT GAC TGG AG
	Reverse TCT GTA GGC TGC CCA ATG AAC

Primer	Sequence 5' → 3'	
SREBP-1c	Forward	GGA GCC ATG GAT TGC ACA TT
	Reverse	GCT TCC AGA GAG GAG GCC AG
SCD1	Forward	ACA CCT GCC TCT TCG GGA TT
	Reverse	TGA TGC CCA GAG CGC TG

Appendix VI-Histological methods

Table A.7: Chemicals and reagents used in fixation, dehydration, embedding and cutting of tissues.

Product name	Vendor
4 % formaldehyde	Merck art. no. 1.04003.1000
NaH ₂ PO ₄ x H ₂ O	Merck art. no. 1.06346.0500
Na ₂ HPO ₄ x 2H ₂ O	Merck art. no. 1.06580.0500
Ethanol (100 %)	Arcus kjemi, Norway
ddH ₂ O	MilliQ Gradient, Lab-tec, Norway
Methanol	Merck art. no. 1.06018.2500
Xylene	Prolabo art. no. 28973.328
Haematoxylin	Chemi-teknik AS, Norway
Eosin Y	Chemi-teknik AS, Norway
Microscopy Entellan	Merck, Germany

Appendix VII-ELISA

Table A.8: Reagents in Insulin Mouse Ultrasensitive ELISA kit for 96 wells.

Product name	Vendor
Insulin Mouse Ultrasensitive ELISA kit:	DRG Instruments GmbH, Germany
Standard 0	Lot no. 17609
Standard 1 (0.175 µg/l)	Lot no. 17599
Standard 2 (0.45 µg/l)	Lot no. 17600
Standard 3 (1.0 µg/l)	Lot no. 17601
Standard 4 (2.5 µg/l)	Lot no. 17602
Standard 5 (6.5 µg/l)	Lot no. 17603
Coated plate	Lot no. 15771
Enzyme conjugate 11X	Lot no. 15878
Enzyme Conjugate Buffer	Lot no. 17606
Wash Buffer 21 X	Lot no. 17557
TMB Substrate solution	Lot no. 15873
Stop Solution 0.5 M H ₂ SO ₄	Lot no. 17561

Appendix VIII - RNA quality and quantification on the Nanodrop ND-100

Table A.9: RNA concentration and A260/A280 and A260/A230 from measurements on nanodrop.

eWAT – Nanodrop measurements					iWAT – Nanodrop measurements				
Mice no.	[RNA] ng/μl	A260	260/280	260/230	Mice no.	[RNA] ng/μl	A260	260/280	260/230
1	1950,34	48,76	2,00	1,77	1	354,56	8,86	1,94	2,19
2	285,08	7,13	1,89	2,07	2	263,98	6,60	1,88	2,22
3	1500,84	37,52	1,99	1,77	3	1210,80	30,27	2,02	2,18
4	391,18	9,78	1,95	2,06	4	363,46	9,09	1,91	2,20
5	332,36	8,31	1,95	2,14	5	417,42	10,44	1,97	2,14
6	302,84	7,57	1,93	2,10	6	854,01	21,35	2,02	2,22
7	224,96	5,62	1,96	2,12	7	493,12	12,33	1,99	2,19
8	830,66	20,77	1,94	2,07	8	1074,97	26,87	2,05	2,20
9	622,21	15,56	2,01	1,71	9	696,54	17,41	2,00	2,20
10	65,19	1,63	1,90	2,19	10	582,83	14,57	1,97	2,24
11	348,61	8,72	1,91	2,19	11	615,92	15,40	2,01	2,00
13	574,12	14,35	1,98	2,20	13	948,28	23,71	2,00	2,20
14	1000,76	25,02	2,01	1,84	14	701,78	17,54	1,95	2,23
15	208,15	5,20	1,84	2,18	15	861,68	21,54	1,99	2,15
16	315,73	7,89	2,04	1,91	16	826,41	20,66	2,07	2,24
17	314,64	7,87	2,05	1,79	17	568,38	14,21	1,97	2,13
19	223,11	5,58	2,03	1,70	19	44,88	1,12	1,83	1,75
20	628,31	15,71	2,07	1,96	20	439,68	10,99	1,98	2,20
21	409,85	10,25	2,03	2,19	21	993,88	24,85	2,02	2,22
22	264,82	6,62	1,94	2,23	22	59,06	1,48	1,84	2,32
23	584,04	14,60	2,08	1,72	23	843,66	21,09	2,00	2,10
24	389,25	9,73	1,92	1,98	24	532,08	13,30	1,97	2,23
25	655,59	16,39	1,98	2,19	25	737,08	18,43	2,07	2,21
26	627,96	15,70	1,97	2,17	26	288,37	7,21	2,01	2,20
27	239,87	6,00	1,99	2,17	27	766,95	19,17	1,97	1,98
28	459,85	11,50	2,03	1,71	28	777,92	19,45	2,05	2,21
29	301,91	7,55	2,00	2,13	29	396,51	9,91	1,95	2,23
30	976,07	24,40	2,05	1,82	30	995,76	24,89	2,04	2,21

Appendix IX- RNA integrity on the Bioanalyzer (RNA 6000 Nano)

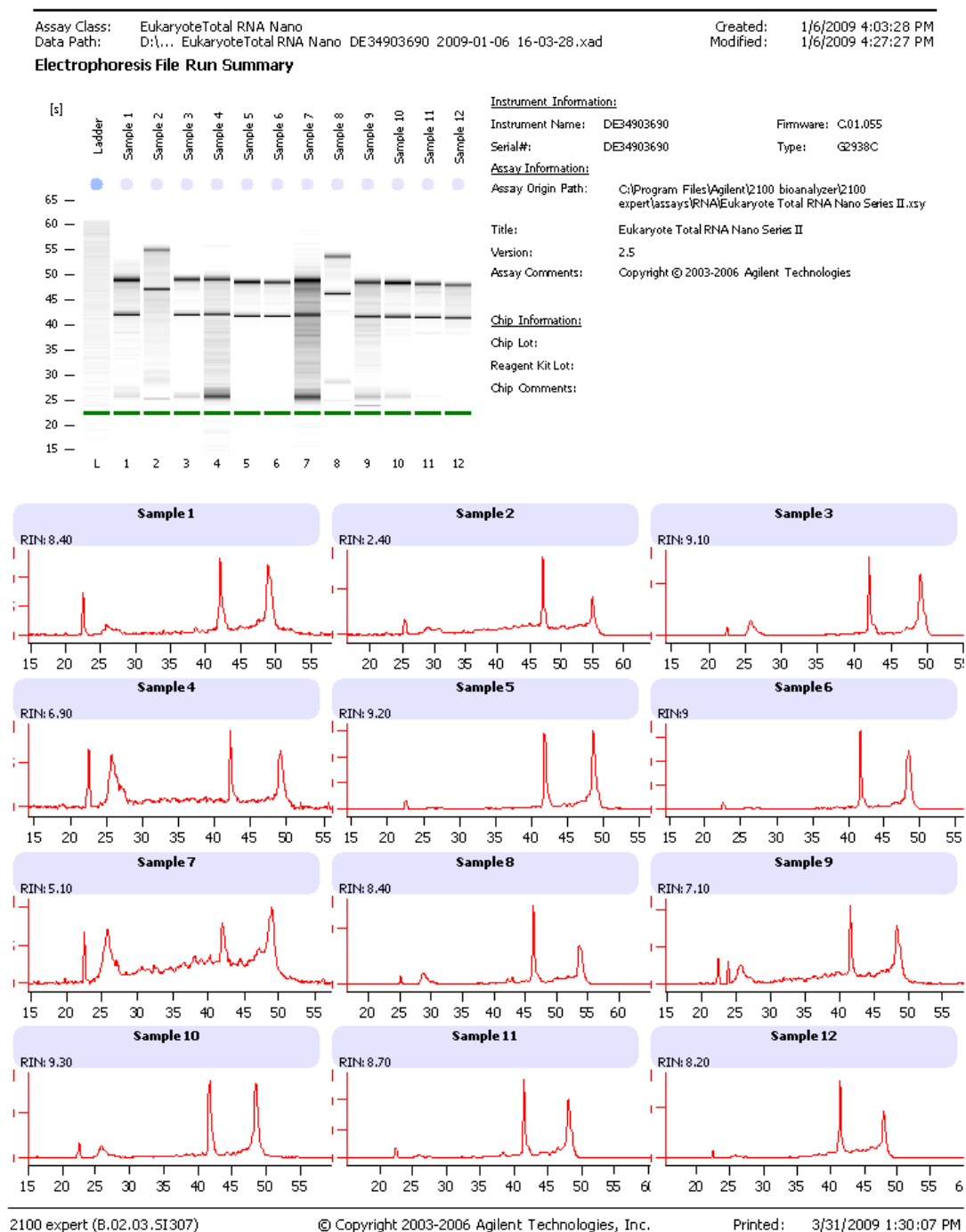


Figure A.1: Results from the Bioanalyzer displayed as a gel-like image and electropherograms. RNA from a selection of adipose tissue (eWAT, iWAT, rWAT and iBAT) samples is presented with RINs.