Gene expression responses to bacterial peptidoglycan in Atlantic salmon (*Salmo salar*) adipocytes enriched with two levels of omega-3 fatty acids and vitamin E

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This thesis I dedicate to my Supervisors,

Dr. Bente Ruyter

Dr. Marijana Todorcevic and Dr. Stanko Skugor

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ABSTRACT

Adipose tissue is recognized as an active endocrine organ, influencing metabolism, inflammation and immunity in mammals. The size of the visceral adipose tissue in farmed Atlantic salmon has increased due to the exposure to lipid-rich formulated diets and immune functions in adipose tissue in Atlantic salmon have been studied with different exposure like lipopolysaccharide (LPS), omega-3 and 9 fatty acids (FAs). However, this thesis has focused on gene expression responses of Atlantic salmon adipocytes to inflammatory inducer peptidoglycan (PGN), different doses of omega-3 polyunsaturated fatty acids and vitamin E. We used primary cell culture of the differentiating adipocytes that harbours the precursors of the white adipocytes to study the gene expression responses of Atlantic salmon adipocytes to different doses of polyunsaturated fatty acids, vitamin E and inflammatory inducer peptidoglycan at Nofima.

Maturing adipocytes isolated from Atlantic salmon were incubated at 13°C until achieving the confluence stage. Cells were treated with with the different doses of omega-3 FAs (0.30 and 0.05 mM) and in addition with either vitamin E (0.1 mM), PGN (10 mM) or vitamin E+PGN 20 days.

Quantitative real time PCR was the preferred approach in this project. We examined the expression level of adipogenic (C/EBPα), fatty acid and energy metabolism (FAS, HSD17B4, PGC-1α, UGT), intracellular antioxidant (GPx-2), immune; pro-inflammatory (TNF-α, STAT1, LECT2, IL-15, IL-1β, IL13a2, ALOX5 and ALOX5AP), anti-inflammatory (RTEL1, IL-1RA, IL-10 and TGF-β), related genes to understand the effects of different mentioned treatments.

Adipogenesis was observed from low omega-3 FAs treatments and vitamin E which turned on synthesis of fat-specific, uptake and storage of long chain fatty acids. Immunostimulant was prone to lower the level of C/EBPα. Moreover, high doses of omega-3 FAs induced lipogenic enzyme (FAS) which influenced to increase with combined treatments of vitamin E and PGN. 17β-HSD4 was induced for inactivation of steroids and β-oxidation by vitamin E

with high doses of omega-3 FAs. In addition, PGC-1α is a transcriptional coactivator for conditional energy metabolism was down-regulated with high doses of omega-3 FAs treatments. UGT which involves in glucuronidation, was induced more with low doses of omega-3 FAs treatments which play role in synergistic effect with low doses of vitamin E. Nonetheless, immunostimulant (PGN) with high doses of omega-3 FAs suppressed induction of FAS, 17β-HSD4, PGC-1α and UGT.

There was no difference between expression level of vitamin E (intracellular anti-oxidant) induction from low and high doses of omega-3 FAs. But significant effect of vitamin E was observed from high+vitamin E treatments which could balance the synthesis of fatty acids. However, vitamin E and PGN showed the opposite effect on adipose cells. The average counter inductions of pro-and anti-inflammatory genes were observed with all treatments. Interestingly, the high expression levels of pro-inflammatory genes were observed in the high+PGN+vitamin E group which was affected by the equally up-regulated expression of anti-inflammatory genes. PGN and high doses of omega-3 showed the highest levels of inflammatory genes expression. However, low doses of omega-3 FAs influenced to suppress induction of pro-inflammatory genes and showed effects on expression of anti-inflammatory genes on average.

In conclusion, low doses of omega-3 FAs with vitamin E have positive effects on adipogenesis. But, vitamin E for inhibition of oxidative damage was more prominent with combined treatments of high doses of omega-3 FAs and vitamin E which was affected by PGN under low omega-3 FAs treatments. All pro-inflammatory genes were down-regualted with low doses of omega-3 FAs treatments and vitamin E helped to induce them. However, high doese of omega-3 FAs induced higher level of anti-inflammatory adipokines which may prevent inflammation from fatty acids syntheis. Importantly, immunostimulant (PGN) was invloed to induce all pro-inflammatory genes except IL-13Rα2 with low+PGN treatments which was counteracted by IL-10, TGF-β and IL-6 simultaneous induction. The significance difference from control was set at $p \leq 0.05$ (t-test).

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Abbreviations

ABCA13	ATP-binding cassette transporter 2
ADD1	Adipocyte determination and differentiation-dependent factor 1
ALOX5	Arachidonate 5-lipoxygenase
ALOX5AP	Arachidonate 5-lipoxygenase-activating protein
ATGL	Adipose triglyceride lipase
BAT	Brown adipose tissue
BMI	Body mass index
C/EBPα	Ccaat/enhancer binding protein α
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CD4	Cluster of differentiation
CRP	C-reactive protein
DEPC	Diethylpyrocarbonate
DHA	Docosahexaenoic acid
EF1A	Elongation factor 1a
EPA	Eicosapentaenoic acid
ESRD	End-stage renal disease
FAs	Fatty acids
FAS	Fatty acid synthase
FFA	Free fatty acids
GPx-2	Glutathione peroxidase-2
HFDs	High fat diets
HSD17B4	17β-hydroxysteroid dehydrogenase type iv
HSL	Hormone-sensitive lipase
HUFAs	Highly unsaturated FAs
IELs	Intestinal intraepithelial lymphocytes
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-13Rα2	Interleukin-13 receptor subunit alpha-2
IL-15	Interleukin 15
IL-1RA	Interleukin-1 receptor antagonist 1
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IMAT	Intermuscular adipose tissue
IU	International unit
LA	Linoleic acid
LECT2	Leukocyte cell-derived chemotaxin 2
LPL	Lipoprotein lipase
LPS	Lipopolysaccharides
mRNA	Messenger ribonucleic acid

MS-222	Metacain
MTP	Microsomal triglyceride transfer protein
OA	Oleic acid
OS	Oxidative stress
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PGC-1α	Peroxisome proliferator activated receptor-γ coactivator-1 alpha
PGN	Peptidoglycans
PKA	Protein kinase A
PPARγ	Peroxisome proliferator-activated receptorγ
qPCR	Real-time quantitative-pcr
RBC	Red blood cells
ROS	Reactive oxygen species
RT	Reverse transcriptase
RTEL1	TNF decoy receptor
SOD	Superoxide dismutase
SREBPs	Sterol regulatory element-binding proteins
STAT1	Signal transducers and activators of transcription 1
TAG	Triacylglycerol
TBARS	Thiobarbituric acid reactive substances
TGF-β	Transforming growth factor beta
TLR1	Toll like receptor1
TNF-α	Tumor necrosis factor-alpha
UGT	UDP-glucuronosyltransferase
VO	Vegetable oil
WA	White adipocytes
WAT	White adipose tissue
α	Alpha
β	Beta
γ	Gamma
δ	Delta
μ	Mu/micro
Δ	Delta

CHAPTER I

INTRODUCTION

Adipose tissue is considered to be an inert organ for energy storage. It has pivotal role in metabolic and immunological process. Endocrine role of adipose tissue is accomplished through the production and release of a large variety of bioactive proteins. Adipose tissue is made of a variety of different cells which play important roles in regulation of its function. However, fat depots in salmonids have increased due to the feeding with formulated diets enriched with lipids. Salmonids are very commercially important species in aquaculture. Adipose tissue is very susceptible with oxidative stress and apoptosis. The doses of omega-3 fatty acids (FAs) have significant role to adjust oxidative damage and balance immune functions. But there is very few omega-3 FAs dose dependent experiment has been done yet on salmonids adipocytes. Todorcevic et al., (2008a) demonstrated that eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) lowered the triacylglycerol (TAG) accumulation in mature salmon adipocytes compared to oleic acid (18:1n-9, OA). Omega-3 FAs are able to change genes expression levels through modulating various transcription factors.

Vitamin E has been accepted as an antioxidant but non-antioxidant property is also reported. Vitamin E is modulator for stress related immunesupression. Vitamin E has preventive measure to inflammatory responses. However, immunostimulant (lipopolysaccharide, LPS and monophosphoryl lipid A, MPL) can induce expression inflammatory genes including TNF-alpha (TNF-α), interleukin IL-1 beta (IL-1 β), type 2 TNF receptor (TNFR-2), and IL-10, D3, D8, and D2 genes (Henricson et al., 1993). Moreover, LPS can increase T lymphocytes and expression of immunomodulatory cytokine, IL-l0 in atopic children (Tulic et al., 2010) and induces expression of immune genes, chemokines and receptors, NFkappa-B related genes, matrix metalloproteinases and genes involved in eicosanoid metabolism in Atlantic salmon (Skugor et al., 2010). Immunostimulant is a potent inducer of immune responses and is widely used in experiments to initiate the inflammatory response. Inflammation is often studied in the LPS-model in mammals (Antonicelli et al., 2004) and a number of

recent findings confirmed that immunostimulant (LPS) is able to trigger strong immune responses in lower vertebrate model (Berczi et al., 1966) as well as in frog (Morera and MacKenzie, 2011) and in fish (Iliev et al., 2005).

Due to the significant effects of fat deposition, fish health, disease and growth are related with adipose biology, our experiment was focused on how different factors influenced gene expression from salmon maturing adipocytes.

Hence, the analytical methods and techniques are explained briefly. Primary cell culture of maturing adipocytes cells was used to isolate total RNA to make cDNA. Total RNA quality and quantity was examined using bioanalyzer and NanoDrop 1000 Spectrophotometer respectively. cDNA was taken for real-time qPCR to measure expression levels of different genes related with adipogenesis, lipogeneis, immunity and energy metabolism.

The main goal of this *in vitro* study was focused on-

1. To examine the gene expression effects of different doses of omega-3 fatty acids, vitamin E and immunostimulant (peptidoglycan) on molecular makers of adipogenesis, lipogensis and energy storage in Atlantic salmon maturing adipocytes.
2. To observe the gene expression effect of omega-3 fatty acids, vitamin E and immunostimulant (peptidoglycan) to pro-and anti-inflammatory responses.
3. To understand defence mechanism of adipose tissue to intra-cellular oxidation.

CHAPTER II

REVIEW OF LITERATURE

2.1. Adipose tissue

Adipose tissue or body fat or fat depot is specialized loose connective tissue. Adipose tissue is conglomerated with connective and nerve tissue, stromovascular cells, macrophages, CD4 (cluster of differentiation 4), CD8 T cells, fibroblasts, endothelial cells, multipotent mesenchymal cells, and immune cells (Ferranate, 2007; Hotamisligil, 1993). It also contains many small blood vessels. The adipose tissue is important to the body in maintaining proper energy balance, storing calories in the form of lipids, mobilizing energy sources in response to hormonal stimulation, and commanding changes by signal secretions. Adipose tissue characteristics often differ between species, between depots within an individual and even between cells in different parts of a depot (Frayn et al., 2003). In mammals, two types of adipose tissue are found, white adipose tissue (WAT) and brown adipose tissue (BAT). Adipose tissue is located under the skin (subcutaneous adipose tissue), between the muscles (intermuscular adipose tissue, IMAT), and around the internal organs (both white and brown adipose tissue). Gregoire et al., (1998) stated, WAT in higher eukaryotes storing triacylglycerol to develop new and effective strategies in controlling periods of energy excess and its mobilization during energy deprivation are its primary purposes.

Liver, muscle and mesenteric fat are considered as lipid storage organs (eg. liver in cod and visceral adipose tissue in salmon) of fish (Sheridan, 1988). Muscle and, especially, the liver are usually proposed as energy storage organs for reproduction (Jonsson et al., 1997) or over-wintering (Morley et al., 2007). According to Fernandez et al., (2009) muscle, liver, testis and ovary serves as energy storage organs in different fish species like *Paranototheni magellanica, P. cornucola, P. sima, P. tessellate, Champsocephalus esox, C. gobio, Eleginops maclovinus, and Odontesthes* sp.

The perception of the role of adipose tissue has changed from a lipid and energy storage organ, as a protector against heat loss, and to serve as a building-block or space-keeper in several organs to an endocrine, metabolic, and immunologically active organ. Adipose tissue play a pivotal role in secretion of a wide variety of hormones and proteins, control in appetite and energy balance, immunity, insulin sensitivity, angiogenesis, blood pressure regulation, lipid metabolism and maintain whole body homeostasis (Zhang et al., 1994) with the invention of leptin in Atlantic salmon and mammals (Vegusdal et al., 2003) and rainbow trout (Murashita et al., 2008) connecting nearly all organs and cell types.

Large amounts of visceral fat in salmon leads to production losses during processing and can significantly influence the health of the fish. Todorcevic et al., (2010b) has shown that the recruitment of new fat cells from unspecialised stem cells and fat cell maturation are controlled by a complex network very similar to the development of human's adipose tissue. The author stated that the composition and functions of the adipose tissue are very important for the health of the fish. It has been known that adipose tissues in salmon secrete hormone-like substances which are important in regulating both energy utilisation and inflammatory reactions (Todorcevic et al., 2010b). In addition to this an increasing degree of lipid accumulation in fat cells leads to increased expression of genes involved in endoplasmic reticulum stress, oxidative stress and inflammation.

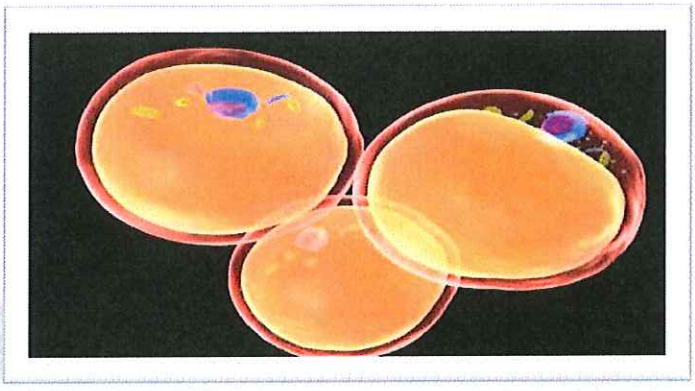

Figure 1: Adipose cells (Moschen, 2006)

2.2. Lipid metabolism of adipocytes

Organisms store energy for later use during times of nutrient scarcity. When energy is required, the stored triacylglycerol is hydrolyzed via activation of lipolytic pathways (Nicole and Perry, 2008). The ability to synthesize sterol esters or triacylglycerols and package these neutral lipids into cytosolic lipid droplets is a universal property of fish and other eukaryotes from yeast to humans.

Triacylglycerols are stored as cytoplasmic 'lipid droplets' (also termed 'fat globules', 'oil bodies', 'lipid particles', 'adiposomes', etc) enclosed by a monolayer of phospholipids and hydrophobic proteins, such as the perilipins in adipose tissue (Williams, 1991).

The primary mechanism by which surplus fuel is made available from adipocytes is through stimulated lipolysis. During lipolysis triglycerides are hydrolysed into free fatty acids (FFA) and glycerol. According to William and Jaswinder (2008) the process liberates FFA and glycerol is physiologically activated by hormones such as catecholamines that stimulate cyclic adenosine monophosphate (cAMP) production and protein kinase A (PKA) dependent phosphorylation of hormone-sensitive lipase (HSL) and perilipins conditions. Perilipins normally inhibit lipolysis by preventing access of lipases to the lipid droplets. In times of energy deficit, perilipin is phosphorylated by PKA and facilitates maximal lipolysis by HSL and adipose triglyceride lipase (ATGL).

Adipose cells lack the enzyme glycerol kinase, some glucose catabolism must occur for triacylglycerol synthesis to take place specifically, the formation of dihydroxyacetone phosphate, for reduction to glycerol 3-phosphate. Glucose acts as a sensor in adipose tissue metabolism; when glucose levels are adequate, continuing production of dihydroxyacetone phosphate generates enough glycerol 3-phosphate for resynthesis of triacylgylcerols from the released fatty acids. When intracellular glucose levels fall, the concentration of glycerol 3-phosphate falls also, and fatty acids

are released from the adipocyte for export as the albumin complex to other tissues (Michael, 2011).

Adipocytes take up glucose, glycerol and FFA from serum and convert these to triglycerides via numerous biochemical pathways. Tumor necrosis factor-alpha (TNF-α) modulates the expression of many of the enzymes and other proteins that regulate these pathways, thereby compromising adipocyte triglyceride storage. TNF-α stimulates lipolysis via a glucose-dependent mechanism that likely involves transcriptional effects. Despite the fact that the major biochemical and metabolic pathways have been identified in fish (Cowey and Walton, 1989), the causal quantitative relationships between dietary energy sources and lipogenesis or muscle fat content are still poorly understood. Available information is limited to a small number of teleost species, but the studies show that in a broad sense, fish are comparable to land animals in terms of lipogenic processes. Fat-rich diets are found to depress activities of several lipogenic enzymes in coho salmon and Atlantic salmon (Lin et al., 1977; Arnesen et al., 1993). High-carbohydrate diets stimulate enzymes involved in fatty acid synthesis in channel catfish and white sturgeon (Likimani and Wilson, 1982; Fynn-Aikins et al., 1992).

2.3. Adipose tissue as a novel endocrine gland

Adipose tissue was a while ago considered as simple, static, lipid-storage tissue. Nowadays, adipose tissue is regarded to be an active and novel endrocrine, paracrine and autocrine organ (Bernardo, 2000). Adipose tissue was first conjectured to have endocrine functions by Siiteri (1987), who pointed to the tissue's ability to interconvert steroid hormones. Adipose tissue is capable of secreting many proinflammatory, anti-inflammatory and immunomodulating biologically active molecules (cytokines, chemokines, and complement factors), immunomodulating adipokines, C1q/TNF-related protein family members, growth factors (Schaeffler et al., 2009; Hotamisligil et al., 1993; Tilg and Moschen, 2006).

Recently interest has moved to adipose tissue production and secretion of a wide range of proteins. Many of these are classical cytokines and others, including leptin, are structurally related to cytokines. In 1994, the discovery of leptin has revealed a new dimension to the understanding of adipose tissue function in mammals (Trayhurn and Wood, 2004). This has led to the introduction of the term adipocytokines to describe this wide range of proteins produced by adipose tissue. In addition, adipocytokine production may differ between adipocytes at different stages of their development, for example, preadipocytes producing less tumour necrosis factor (TNF) than mature cells (Frayn et al., 2003).

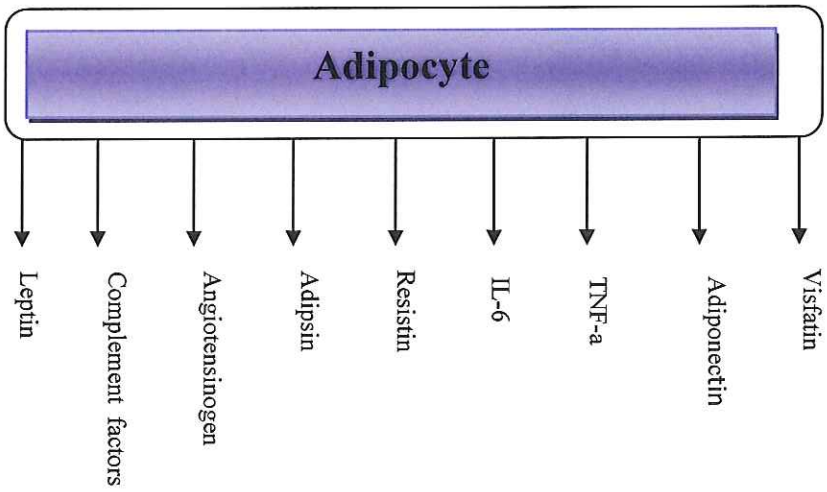

Figure 2.1: Adipose derived peptides

Recent microarray study reveals gene expression profiles (lipid metabolism, adipogenic, carbohydrate metabolism, cell cycle, immune related, protein folding and translation, calcium regulated, endoplasmic reticulum stress and antioxidant system) in Atlantic salmon during the differentiation of its preadipocytes *in vitro* (Todorcevic et al., 2010a).

Recent studies suggest that adipose tissue inflammation is due in large part to the pro-inflammatory actions of bone marrow-derived WAT macrophages. Macrophages are monocytic phagocytes that function in innate immunity and wound-healing by

sequestering and clearing pathogens, dead cells and cell debris in an activation-dependent manner (Duffield, 2003). Macrophage numbers and/or macrophage inflammatory gene expression in WAT are positively correlated with adipocyte size and body mass index (BMI) in mice and negatively correlated with weight loss in obese humans (Clement et al., 2004; Weisberg et al., 2003; Cancello, 2005). While in case of fish relation between quantity and inflammatory genes expression of macrophage is not studied much yet. Macrophages are the predominant source of TNF-α (Todorcevic et al., 2010a), and a significant source of interleukin-6 (IL-6) in WAT of obese (*ob/ob, db/db*) mice and humans (Xu, 2003; Fried et al., 1998 and Fain et al., 2004). Intimate relationship between metabolic regulation and immune responses in WA (White Adipocytes) are found in Salmon a cold-blodded vertebrate (Todorcevic et al., 2010a).

2.4. Fate of excessive lipid storage in adipose tissue

Excess lipid accumulation in adipose tissue can lead to load lipids in non-adipose tissues eg. heart, skeletal muscle, pancreas, liver, and kidney may arise in the setting of high plasma free fatty acids or triglycerides. Surplus lipids may eventually turn into the pathogenesis of heart failure, obesity, impair normal cell signaling, causing cellular dysfunction and induce apoptotic cell death. The process, termed as lipotoxicity, can account for many manifestations of the 'metabolic syndrome'. Moreover, abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, insulin resistance or glucose intolerance, prothrombotic state and proinflammatory state are considered as metabolic risk factors for human (Scott et al., 2004). Nonetheless, the evidence and effects of metabolic syndrome in case of fish is still undisclosed. Mitochondrial damage may be caused by oxidative damage, which is closely related with lipid accumulation (Schaffer, 2003). However, little has been gained about causing factors explaining excess fat accumulation and their proximate diseases in Salmoinds. Steatohepatitis (Thomsen et al., 1994), cardiomyapathy and eventual heart failure (Herpen et al., 2008) in mammals is prominent due to abnormal fat synthesis where in case of fish is unrevealed.

However, coronary arteriosclerosis in salmonids develops in immature fish, well before maturation, and progresses with age and reveals that coronary arteriosclerosis is possible factor of heart disease in fish (Farrell, 2002). Dietary factors, especially polyunsaturated FAs (and their metabolites), can significantly stimulate vascular smooth muscle proliferation in the salmon coronary artery. However, coronary lesions and blood flow to the salmon heart has not been properly studied (Farrell, 2002).

The functions of lipogenesis and lipolysis can be regulated by numerous extracellular stimuli, such as insulin, cortisol, catecholamines, growth hormone, testosterone, FFA and cytokines. TNF-α action on adipocytes can directly alter lipid metabolism through inhibition of FFA uptake and lipogenesis and stimulation of FFA release via lipolysis. In this way, adipose tissue-derived TNF-α can contribute to the development of dyslipidaemia and resultant metabolic complications. In obesity or excess adipose tissue in mammals reveals low-grade inflammatory stresses (Cancello and Clement, 2006 and Maachi et al., 2004) which play vital role with production of pro-inflammatory (Toll like receptor; TLR1–TLR9, IL-6 receptor, TNF receptor), anti-inflammatory (Taniguchi and Yamamoto, 2005), and immunomodulating biologically active molecules, accompanied with metabolic syndrome (Mehta and Reilly, 2004) where fish is still subject of research interest.

2.5. Effects of omega-3 fatty acids on Atlantic salmon adipocytes

Effects of omega-3 FAs on Atlantic salmon adipocytes have been started to know mostly from Marijana PhD experiments in 2008 (Todorcevic et al., 2008a). Marijana Todorcevic who started the first work on development and functions of adipose tissue in Atlantic salmon (Todorcevic et al., 2008a). Highly unsaturated FAs (HUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) prevent the development of obesity in mammals by reducing the growth of visceral adipose tissue (Ruzickova et al., 2004). Omega-3 FAs inhibit the late phase of adipocyte differentiation through the suppression of peroxisome proliferator-activated receptor α and CCAAT/enhancer binding protein γ (PPARγ and C/EBPα) two major genes that regulate terminal differentiation (Madsen et al., 2005). Other proposed mechanisms

are that HUFAs suppress FA synthesis and /or induce mitochondrial FA β-oxidation, leading to the utilization of stored lipids and a reduction in adipocyte size (Flachs et al., 2005). However, it has recently been suggested that the loss of adipose tissue may also be caused by a decrease in adipocyte number, and that this occurs by, for example, apoptosis (Kim et al., 2006) induced by oxidative stress. Adipocytes cultivated in omega-3 FAs enriched medium has lower TAG levels than cells cultivated in a medium supplemented with oleic acid (OA) (Todorcevic et al., 2008a). Todorcevic et al., (2008a) demonstrated that HUFAs affect FA utilization, fat storage and oxidative stress (OS) in Atlantic salmon (*Salmo salar*) WAT. However, increased levels of omega-3 FAs resulted in lower fat percentage in WAT in salmon (Todorcevic et al., 2010b). With the increasing dietary levels of these omega-3 FAs, the levels of DHA and EPA in phospholipids (PLs) from WAT and mitochondrial membranes increased and generally mitochondrial membrane PLs are characterised by lower levels of omega-3 FAs and higher levels of linoleic acid (LA; 18:2 n-6) than WAT membrane PLs. The higher level of LA relative to omega-3 FAs in mitochondrial membrane PLs may help to protect these PLs from peroxidation. The increase activity of superoxide dismutase (SOD) in the DHA and EPA groups results in OS in fish fed with high dietary dose of DHA and EPA whereas the increased activity of caspase-3 in the high omega-3 FAs groups indicates the induction of apoptosis and increased incidence of cell death in WAT.

It is known that EPA and DHA act as a mitochondrial proliferators and increase mitochondrial β-oxidation activity in fish muscle and liver cells (Kjaer et al., 2008). Increasing levels (11–58% of dietary total FAs of HUFAs has an effect on muscle and mitochondrial membrane lipids and their susceptibility to oxidative stress of Atlantic salmon (Østbye et al., 2009). According to Østbye et al., (2009) EPA and DHA groups contain lower percentages of sphingomyelin and cardiolipin in total muscle than the intermediate omega-3 FAs group and this cardiolipin and sphingomyelin are particularly susceptible to peroxidation. A lower percentage of these high lipids indicate oxidative damage of mitochondrial membranes whereas the intermediate n-3 HUFA group has the highest level of mitochondrial integrity and tendencies of lower

level of thiobarbituric acid reactive substances (TBARS). A higher degree of myofibre–myofibre detachment is found in fish fed the high omega-3 FAs diets than in fish fed the intermediate omega-3 FAs diet. It is found that intermediate levels of omega-3 FAs in salmon diets are best for protection against oxidative damage of mitochondrial membranes and muscle structure.

Hong et al., (2006) determined the effects of omega-3 FAs in grass carp (*Ctenopharyngodon idellus*) and stated that weight gain, specific growth rate, feed efficiency and protein efficiency increased by increasing the dietary HUFAs content from 0% to 0.52%, and declined thereafter. However, excess HUFAs fortification may exert adverse effects, which might be due to oxidative stress.

Ling et al., (2006) showed that female swordtail (*Xiphophorus helleri*) benefits from inclusion of dietary HUFA during reproductive stages, despite possessing the ability to increase transcription of desaturase and elongase during low dietary HUFA provision. However, excessive dietary HUFA could result in imbalanced ratios of HUFA, which could impair reproductive processes.

Preadipocytes represent a wide variety of cells that have the potential to either proliferate or differentiate into lipid-assimilating cells found within fat tissue (Hausman et al., 2001; Kokta et al., 2004; Novakofski, 2004; Fernyhough et al., 2007). Omega-3 FAs or their metabolites (Duplus et al., 2000, 2001) have effects on proliferation and differentiation of these cells (Amri et al., 1994). Gene expression is affected by omega-3 FAs through working with transcription factors such as PPARs. However, an alternatively spliced form of PPARγ has been discovered in Atlantic salmon adipocytes (PPARγ short) (Todorcevic et al., 2008a). Stimulation of PPARγ by fatty acids or specific ligands such as the fibrates, results in increased fatty acid oxidation (Kliewer et al., 1997; Wolfrum et al., 2001). PPARγ2 is highly expressed in WAT and its stimulation by omega-3 FAs (Tontonoz et al., 1994; Gregoire et al., 1998; Thuilier et al., 1998). In porcine preadipocyte cultures, omega-3 FAs is evolved with gene expression of both transcription factors and markers of adipocyte differentiation (Ding et al., 2001a,b). However, a clear relationship of fatty acids and

transcription factor expression in relation to adiposity *in vivo* has not been established (Ding et al., 2003).

Polyunsaturated fatty acids (PUFAs) are known to suppress lipogenic gene transcription by downregulating the expression of the sterol regulatory element-binding proteins (SREBPs) (Worgall et al., 1998; Xu et al., 1999) and they may function as activators/ligands for the PPARs (Barak et al., 1999; Forman et al., 1997 and Etgen et al., 1997).

It is well documented that diets enriched in n-3 PUFAs decrease adipose growth in rodents (Shimomura et al., 1990; Wang et al., 2002; Minami et al., 2002 and Cha et al., 2001). However, high level of HUFAs in the diet decreases the amount of accumulated fat in adipose tissue. In addition, the amount of stored fat is decreased when adipocytes are treated with HUFAs *in vitro* (Todorcevic et al., 2008b). Moreover, omega-3 FAs limit the development of visceral adipose tissue by suppressing the late phase of adipocyte differentiation through modifications of PPARγ (Ide et al., 1994). Nevertheless, EPA, and not DHA, lowers plasma triacylglycerol by increasing mitochondrial fatty acid oxidation and decreased availability of fatty acids for triacylglycerol synthesis (Madsen et al., 2005). However, energy balance and body fat content can be influenced by changing the dietary omega-3 FAs level (Couet et al., 1997 and Pan et al., 1995).

Todorcevic et al., (2008a) demonstrated that EPA and DHA lower the triacylglycerol (TAG) accumulation in mature salmon adipocytes compared to oleic acid (18:1n-9, OA). This finding indicates that a reduced level of HUFAs in fish diets, when the traditional marine oil is exchanged for n-9 FAs rich vegetable oils (VOs), may influence visceral fat deposition in salmonids. Moreover, major differences in the metabolism of EPA, DHA and OA at different stages during differentiation of adipocytes occur. Most of the EPA and DHA are oxidized in preadipocytes, while they are mainly stored in TAGs in mature adipocytes in contrast to OA which is primarily stored in TAGs at all stages of differentiation. However, due to fish oil (FO)

shortage, we decided to test the effects of two different concentrations of omega-3 FAs and their effects on adipocytes.

2.6. Effects of vitamin E (α-tocopherol) on adipocytes

Vitamin E (α-tocopherol), the most important lipid-soluble antioxidant, and thus it must be provided by foods and supplements (Burton, 1998), is discovered at the University of California at Berkeley in 1922. The eight isomers of vitamin E are widely distributed in nature. Vitamin E has been detected in varying compositions (4±160 mg/g fresh weight) in all plants having been examined so far (Threlfall, 1971). The significance of vitamin E is subsequently proven as a radical chain breaking antioxidant that can protect the integrity of tissues and play an important role in life processes (Traber, 1990). Since its discovery, studies of the constituent tocopherols and tocotrienols have focused mainly on their antioxidant properties. More recently α-tocopherol is found to possess functions that are independent of its antioxidant/radical scavenging ability.

Vitamin E is one commonly used antioxidant supplement in fish diets, but the mechanisms of its action in fish are not fully understood (Mourente et al., 2002). Vitamin E is proven to prevent OS to a certain extent, but it has antioxidant effects that are independent of glutathione (Todorcevic et al., 2010b). Vitamin E seems to be a potent pro-adipogenic vitamin, as it was associated with the up-regulation of the central transcription factors that are essential for the terminal differentiation of adipocyte in Atlantic salmon (Todorcevic et al., 2010b). However, vitamin E has effects on weight gain, feed efficiency and other nutritional indices in rohu fry (Sau et al., 2004).

Vitamin E helps to reduce production of prostaglandins such as thromboxane, that cause platelet clumping. Thromboxane is formed from arachidonic acid, which is high in western diets (Packer, 1991). Vitamin E also acts as a cell membrane stabilizer, which is postulated by some researchers to be the primary mechanism for its prevention of muscle damage (Meydani et al., 1996; Tiidus and Houston, 1995).

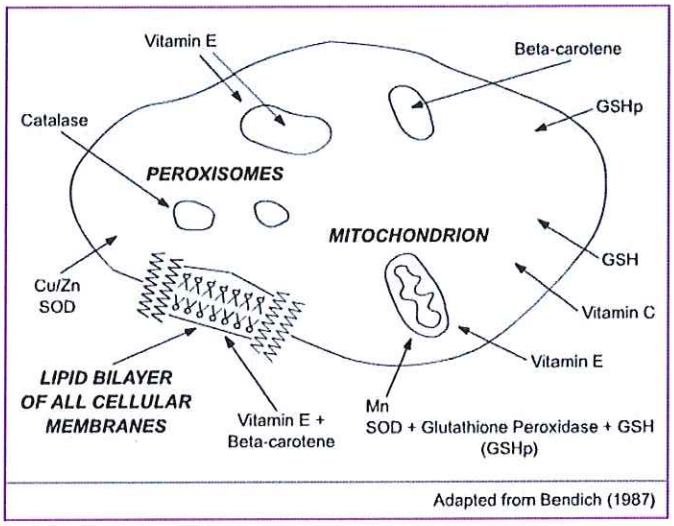

Figure 2.2: Antioxidant system (Bendich, 1984)

In 1991 Angelo Azzi's group (Boscoboinik et al. 1991) first described non-antioxidant cell signalling functions for α-tocopherol, demonstrating that vitamin E regulates protein kinase C activity in smooth muscle cells. Absorption in the body is α-tocopherol selective and other tocopherols are not absorbed or are absorbed to a lesser extent. Furthermore, pro-oxidant effects are attributed to tocopherols as well as an anti-nitrating action (Azzi and Stocker, 2000). Activation events are found on the protein phosphatase PP2A and on the expression of other genes (α-tropomyosin and connective tissue growth factor) (Azzi and Stocker, 2000). In cultured cells it has been demonstrated that vitamin E inhibits inflammation, cell adhesion, platelet aggregation and smooth muscle cell proliferation. Recent advances in molecular biology and genomic techniques have led to the discovery of novel vitamin E-sensitive genes and signal transduction pathways.

It is well known that high fat diets (HFDs) increase in proinflammatory adipokines. IL-6 is considered the major inflammatory mediator in excess fat mass. IL-6 protein levels and the IL-6/IL-10 ratio are decreased in epididymal white adipose tissue by

receiving vitamins E supplementation. Vitamin E can significantly reduce the IL-6 levels in mice and in 3T3-L1 adipocytes stimulated with LPS without affecting IL-10 levels (Fabio et al., 2011). However, adipokines stimulation in response with with either vitamin E or along with immune-stimulant is still unexplored in case of fish. Vitamin E intakes reduce plasma C-reactive protein (CRP) and IL-6 concentrations in diabetic subjects (Upritchard et al., 2000).

The effect of α-tocotrienol can able to decrease the insulin-induced PPARγ mRNA expression by 55% compare with insulin, whereas α-tocopherol increase the mRNA expression (Uto-Kondo et al., 2009). Supplementation of vitamin E restores the concentrations of adipocyte determination and differentiation-dependent factor 1 (ADD1) and FAS mRNA, which is decreased by DHA treatment (Liu et al., 2005).

Vitamin E has independent positive antioxidant effects on adiponectin mRNA and protein secretion in 3T3-L1 adipocyte cells (Landrier et al., 2009). Vitamin E up-regulates adiponectin expression via a mechanism that implicates PPARγ together with its endogenous ligand 15-deoxy-Delta 12, 14-prostaglandin J2 (Landrier et al., 2009). Besides being a very important natural antioxidant, α -tocopherol also acts as a significant nonantioxidant. Furthermore, the nonantioxidant action of α-tocopherol appears to be of particular relevance at the cellular level (Chatelain et al., 1993; Boscoboinik et al., 1991).

The increasing susceptibility of highly unsaturated membrane phospholipids to free radical initiated oxidation, vitamin E and selenium play the significant role in preventing initiation and propagation of the lipid peroxidation (Valk and Hornstra, 2000).

Cellular and molecular studies provide a possible mechanism whereby by vitamin E may exert its action, namely by scavenging peroxy-radicals and thereby acting as a chain-breaking antioxidant (Burton and Ingold, 1981; Serbinova and Packer, 1994 and

Witting et al., 2000). However, vitamin E protects PUFAs and protein thiol groups against oxidation (Ferre et al., 2001 and Scaccini et al., 1992).

The content of PUFAs in adipose tissue negatively correlates with vitamin E content in that tissue. However, plasma vitamin E levels decrease upon long term fish oil supplementation and that this effect is alleviated by higher dietary vitamin E intake (Cho and Choi, 1994; Javonhey-Donzel et al., 1993 and Pallozza and Krinky, 1992).

The vitamin E requirement in farmed Atlantic salmon (Salmo *salar)* varies extensively with different experimental conditions. The diet contained 17% lipid and 5.6% PUFA, which corresponds well with lipid and PUFA levels currently used in practical diets for salmon fry.

An increase in vitamin E requirement caused by increased dietary PUFA has been confirmed in fish farming (Watanabe, 1982) and in mammals (Witting and Horwitt, 1964).

The level of dietary PUFA is increased due to an increase in vitamin E requirements and the enrichment of tissues with α-tocopherol leads to less oxidation of fatty acids in tissues subjected to activated oxidation, even if these tissues contain rather high levels of *n-3* PUFA (Leibowitz et al., 1980).

However, in mammals, the protection of PUFA from peroxidation depends on the tissue (Leibowitz et al., 1980; Monahan et al., 1992). In fish, high levels of PUFA induce higher α-tocopherol requirements (Watanabe et al., 1981; Cowey, 1993).

A dietary α-tocopherol supplement partly inhibits the oxidative processes responsible for muscle lipid darkening during cold storage of freshwater fish such as channel catfish (0' Keefe and Noble, 1978; Gatlin et al., 1992), rainbow trout (Boggio et al., 1985; Frigg et al., 1990) and also in a marine fish, the sea bass (Stephan and Lamour, 1993). Dietary a-tocopherol in broiler diets has long been accepted as a feasible means

of delaying lipid oxidation and prolonging shelf life (Combs and Regenstein, 1980; Bartov and Bornstein, 1981). The vitamin E requirement of certain terrestrial animals and of certain species of fish is affected by the quantity of PUFA in the diet and by their degree of unsaturation.

Belo et al., (2005) suggests that vitamin E may contribute to the efficiency of the fish's inflammatory response by increasing macrophage recruitment and giant cell formation in the foreign body granulomatous reaction. Vitamin E appears to act on the stress response of pacus fish by preventing a stress-related immunosuppression.

Amlashi et al., (2011) indicates that vitamin E had no significantly effect on muscle proximate analysis, hematological and immunological parameters of sub-yearling beluga (*Huso huso*) but has a direct effect on growth performance of beluga sturgeon and this vitamin is an essential nutrient required for normal growth in this species.

Vitamin E could modulate adiponectin expression in adipocytes, which could constitute an additive mechanistic explanation for the effect of vitamin E on adipocytes. This vitamin is synthesized by plants, and has eight different isoforms (vitamers) divided into two classes of four vitamers each. Vitamin E is integral part of cellular membranes whose main role is to protect the cell against oxidation. Within cells and organelles (e.g. mitochondria) vitamin E is the first line of defence against lipid peroxidation. Vitamin E also plays a very important function in lending red blood cells (RBC) flexibility as they make their way through the arterial network.

Concentrations of vitamin E cover a wide range in body tissues. The actual mechanisms regarding vitamin E release from the tissue is unknown till now. While it seems likely that vitamin E is released during lipolysis associated with exercise this may not be true and even during times of weight reduction vitamin E is not released from the adipose cells (Traber, 1999). Therefore, the factors that regulate bioavliability of vitamin E from adipose tissue are not known.

Vitamin E is excreted mainly via bile, urine, feces, and the skin. It is oxidized and forms hydroquinone and then is conjugated to form glucuronate. Once formed the glucuronate can be excreted into bile or further degraded in the kidneys and excreted in the urine.

2.7. An immunostimulant

Immunostimulants are chemical compounds that instigate white blood cells and affirm animals to infections by viruses, bacteria, fungi and parasites. In the presence of chemical signals the immune system respond as if challenged by a pathogenic microbe. Hence, administration of an immunostimulant prior to an infection may elevate the defence barriers of the animal and thus provide protection against severe or lethal infection.

Bacteria cell wall (e.g. lipopolysaccharides (LPSs), lipopeptides, peptidoglycans (PGN) and muramyl peptides) are very potent immunostimulants.

Immunostimulants may act in synergy with antibiotics and thus strengthen the effect of curative medication. However, immunostimulants are primarily prophylactic agents which should be used to-

- Elevate the general defense barrier of the organism and hence to reduce the risk of disease, not as a curative medicine.

2.8. Role of immunostimulant

Immunostimulants are being used today both within the aqua-culture sector and in traditional animal husbandry to reduce mortality due to infections and to improve general performance of animals including fish and shrimp in aquaculture. LPS and PGN helps to activate innate immune cells such as macrophages and adaptive immune B cells (Li and Qin. 2005). The interaction between adipocytes and macrophages results in marked upregulation of pro-inflammatory adipocytokines and significant downregulation of anti-inflammatory adipocytokines (Schenk et al., 2008).

Immunostimulants may provide particular benefits when used in order to (Jan R, 2000):

- Prevent virus diseases
- Enhance disease resistance of farmed shrimp
- Reduce mortality of juvenile fish
- Enhance the efficacy of anti-microbial substances
- Enhance the efficacy of vaccines
- Enhance the resistance to pathogenic micro-organisms, parasites and reduce mortality due to opportunistic pathogens e.g. spring and autumn blooms in the marine environment, high stocking density (Jan R, 2000).
- Develop general performance in stress and impaired of animals e.g. handling, change of temperature and environment, weaning of larvae to artificial feeds (Jan R, 2000).
- Enhance developmental phases when animals are particularly susceptible to infectious agents e.g. the larvae phase of shrimp and marine fish, smoltification in salmon, sexual maturation (Jan R, 2000).

Improvements in the health status of fish can certainly be achieved by balancing the diets with regard to nutritional factors, in particular lipids and antioxidative vitamins, but this is primarily a result of an input of substrates and cofactors in a complex metabolic system. This is unlike immune-stimulants, which interact directly with the cells of the immune system and make them more active.

Eicosanoid metabolism influences expression of immune genes, including TNFα and TNF-dependent genes, chemokines and receptors exposure to LPS in Atlantic salmon (Skugor et al., 2010). Immunostimulants is able to accelerate a number of transcriptional events during the adipogenic differentiation and decrease expression of adipocyte markers and genes involved in lipid metabolism of preadipocytes (Skugor et al., 2010).

Immunostimulants treatment of isolated primary cells, (Ajuwon et al., 2004) and established mouse 3T3-L1 (Rodriguez-Calvo et al., 2008) cell cultures containing adipocytes at different stages increases production and release of proinflammatory cytokines (TNFa and IL-6) and chemokines and expression immune genes stimulate NFkappaB down-regulation and ligand induced activation of PPARγ (Qiao et al., 2008). Immunostimulants (LPS) decreases expression of adipogenic markers (eg. PPARγ, C/EBPα, C/EBPδ) and proteins of lipid metabolism and activates lipolysis (Skugor et al., 2010). Metabolic responses of fish adipocytes can be influenced by LPS (Albalat et al., 2005).

Immunostimulants is able to decrease activity of extracellular superoxide dismutase SOD (Adachi et al., 2005). SOD, is an important scavenger of reactive oxygen species (ROS). ROS is an anti-adipogenic in mammals (Carriere et al., 2004) and Skugor et al., (2010) confirms the negative role of ROS in lipogenic pathways in Atlantic salmon. LPS has down-regulation influence of PPARγ, a key regulator of adipogenic development and lipogenic pathways, consistent with the down-regulation of lipogenic markers (eg. microsomal triglyceride transfer protein, MTP and ATP-binding cassette transporter 2, ABCA13) (Skugor et al., 2010). Immunostimulants can enhance lipolysis in large murine adipose depots (Berkowitz et al., 1998).

2.9. Cell Culture Systems

The use of in vitro cell cultures has provided a great deal of information closely mimicking the in vivo state. The cell culture systems have provided tools to study biochemistry and molecular biology. Like mammalian cells, fish cells can be maintained in vitro in two ways: cell lines and primary cultures. Most of biological experiments are performed with immortalized cell lines since they are readily available and can be expanded without limitation. Established cell lines present a homogenous cell population and can be carried in culture indefinitely. Cell that are cultured directly from animal tissue are known as primary cells. Primary cultures can keep the differentiated state for a short period and have limited life span. Primary cell culture offers a more relevant system for the study of cell function, disease states.

Primary cell cultures have the advantage in comparison to cell lines, that environmental conditions can be precisely controlled. However, working with primary cell in culture presents numerous challenges, including the requirements for unique cell supplements and growth conditions.

An important advantage of primary cells, in comparison to cell lines, is that they more closely resemble the in vivo situation, since they represent heterogeneous population of cells. However, in vitro systems share the characteristics that they exclude the influence of other organs and of the circulatory and immune system.

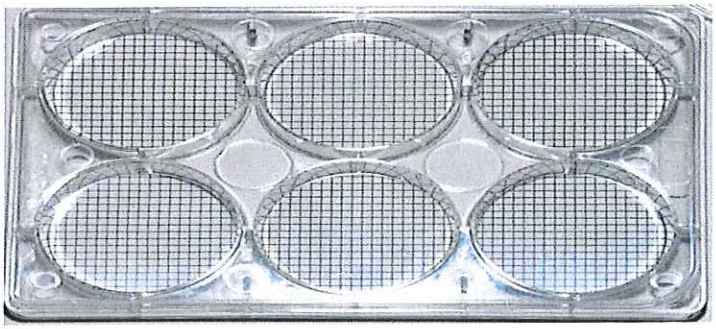

Figure 2.3: Cell Culture Plates

CHAPTER III

Materials and Methods

A study was carried out from 01 November 2010 to August 2011 to analyze the gene expression responses of Atlantic salmon adipocytes to inflammatory inducer peptidoglycan (PGN), different doses of omega-3 polyunsaturated fatty acids (FAs) and vitamin E. The details materials and methods for the experiment are presented in this chapter.

3.1. Materials

Atlantic salmon (*salmo salar*) were obtained from NOFIMA's Research Station, Trømso, Norway. Fetal bovine serum (FBS), antibiotics (combination of penicillin, streptomycin and amphotericin B), 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), lipid mixture, laminin, Thermanox cover slips, phosphate buffered saline solution (PBS), Leibowitz-15 (L-15) were used as materials. Metacin (MS-222) was obtained from Nosk Medisinaldepot (Oslo, Norway). Cell wall components from bacterium (PGN-EB; Peptidoglycan from *E. coli 0111:B4)* was bought from InvivoGen. Collagenase (type I, 220U/mg) was obtained from Worthington (Lakewood, NJ, USA). RNeasy Mini Kit, QIAshredder columns and RNase-free DNase I were purchased from Qiagen (Vaencia, CA, USA). TaqMan RT-PCR Kit was bought from Applied Biosystems (Foster City, CA, USA). NanoDrop 1000 Sectrophotometer (Thermo Fisher Scientific Inc.), Agilant 2100 Bioanalyzer (Sr. No. DE72901876) and Light Cycler 480 (Roche Diagnostics, Mannheim, Germany) were used.

3.2. Isolation and culture of adipocytes

Atlantic salmon (*Salmo salar*) were fed commercial diet prior to sampling. Fish were anesthetized with metacain (MS-222). The arch bows of gills were cut and after bleeding for 1-2 minutes, the fish were killed by a blow to the head. The abdomen was cut and opened to expose the visceral adipose depot. Intestinal fat was removed carefully to avoid cross-contamination with intestinal contents. In addition, the fat was rinsed in petri dishes with L-15 until there was no visible of blood.

The fat tissue was transferred into 50 ml tubes containing L-15 and kept on ice until all fish were dissected. The weights of tubes were taken before putting the fat into them to keep control of the amount of collected fat. Then the fat tissue was cut into small pieces and

washed twice with L-15. The tissue was centrifuged at 280g for 5 minutes prior to transferring it with a spoon into new tubes with L-15. The buffer was decanted off and the tissue was resuspended in new buffer and transferred again into new tubes with L-15. Tissue was then centrifuged at 1250 rpm for 5 minutes and transferred into 0.1% collagenase (Type 1) solution (1 g tissue per 5 ml L-15). Collagenase digestion was executed by gentle shaking using electric stirrers for 1 hour at 13°C. The digested tissue was filtered through 250 and 100 µm nylon filters into 50 ml tubes to remove large particulate material and rinsed through the filter with L-15. The filtered was then centrifuged at 700g for 10 minutes. Then the cells were precipitated to the bottom of tubes and the digestion buffer was decanted off. The precipitated cells were washed twice in L-15 medium and then centrifuged at 700g for 5 minutes where L-15 was decanted off again. The cells were resuspended in growth medium containing 440 ml L-15, 5 mM HEPES, antibiotics and 10% fetal bovine serum (FBS) and then finally centrifuged at 700g rpm for 10 minutes prior to decanting off the medium and plated the cells. Prior to plating the cells, each well was coated with 15 µl laminin and dried for about 5 hours. The cells were incubated at 13°C. Cells were washed every three with L-15 medium until achieving the confluence stage. Cells were treated with different treatments for 6 days. The cells first got treatments with FAs and vitamin E. Then second treatments were given after three days with (including FAs and vitamin E) PGN.

Table 3.1. Concentration of different treatments of Eicosapentaenoic acids (EPA), Docosahexaenoic acid (DHA), Vitamin E (α-TOC) and Peptidoglycan (PGN).

Well number	Final conc.(mM) EPA	Final conc.(mM) DHA	Final conc.(mM) Vitamin E	Final conc.(mM) PGN	Rename
1-6	0.3	0.3	-	-	high Omega
7-12	0.3	0.3	0.1	-	high omega + vitamin E
13-18	0.3	0.3	-	10	high omega + PGN
19-24	0.3	0.3	0.1	10	high omega + vitamin E + PGN
25-30	0.05	0.05	0.1	-	low omega + vitamin E
31-36	0.05	0.05	-	10	low omega + PGN
37-42	0.05	0.05	0.1	10	low omega + vitamin E + PGN
43-54	0.05	0.05	-	-	low Omega

3.3. RNA extraction

Adipocytes were washed two times with PBS. RNA isolation was carried out by using an RNeasy Mini Kit. RLT buffer (lysis buffer for disrupting and denaturing cell) was mixed with 1% ß-mercaptoethanol which helps to inhibit RNase. 300 µl of RLT buffer was added to each cell well. Further, cells were scraped off and cell suspension was transferred to QIA-shredder columns and centrifuged at 15700 x g for 2 minutes. The lysate, which load onto the upper part of the columns, was thrown away. Then, 1 x volume (300 µl) of 70% EtOH (70% ethanol) was added to the suspension in the bottom of the tube, mixed and transferred to RNeasy columns. After that, the columns were centrifuged at 15700 x g for 15 seconds.

350 µl of RWI buffer (washing buffer) was added and spin at 15700 x g for 15 seconds. Further, DNase mix was prepared by mixing 10 µl of DNase I stock solution and 70 µl of RDD buffer per sample. It was mixed through inverting. 80 µl of DNase incubation mix was added to each colon and then incubated for 15 minutes at room temperature. After that, 350 µl of RWI buffer was added again and centrifuged at 15700 x g for 15 seconds.

500 µl of RPE buffer with ethanol (washing buffer) was added and centrifuged on 15700 x g for 2 minutes. Again it was added 500 µl of RPE buffer and centrifuged on 15700 x g for 2 minutes. Then the colons were transferred to a new tubes and spin at 15700 x g for 1 minute. Finally the colons were moved to 1.5 ml eppendorf tube and eluted with 40 µl RNase-free water. Samples were centrifuged at 15700 x g for 1 minute. The same elution processes was done again by transferring the content onto colon once more and spin again at 15700 x g for 1 minute.

3.4. RNA quality assay

Concentrations of each sample were assessed with the NanoDrop 1000 Sectrophotometer. While the quality of RNA was determined by using bioanalyzer which is widely used for determing the quality of RNA before running microarray or real time quantitative PCR experiments. Agilant 2100 bioanalyzer (Sr. No. DE72901876) was used for total RNA or mRNA quality analysis. 550 µl of RNA 6000 Nano gel matrix (Red marked) was pipetted into a spin filter prior to centrifuge at 1500g for 10 minutes at 22°C. Moreover, 65 µl of filtered gel was aliquoted into 0.5 ml RNase-free microfuge tubes. Then, RNA 600 nano dye concentrate (blue maked) was equilibrated at 22°C for 30 minutes prior to vortex for 10 seconds. For preparing the gel-dye mix, tube was taken for spinning at 13000g for 10

minutes at 22°C. To load the gel-dye mix, a new RNA 6000 Nano chip was put on the chip priming station for preparing the gel-dye mix prior to pipette 9.0 µl of gel-dye mix in the well-marked G where the plunger was positioned at 1 ml and then closed the chip priming station. Plunger was pressed until it was held by the clip and after 30 seconds clip was released prior to pµll back plunger to 1 ml position after 5 seconds. Then, chip priming station was opened and pipetted 9.0 µl of gel-dye mix in the wells marked G. The leftover was discarded. 5µl of RNA 6000 nanomarker (green marked) was pipetted in all 12 sample wells as well as in the well-marked ladder to load the nano marker. Ladder and samples were denatured for 2 minutes at 70°C to get primary structure as it runs well in gel. Moreover, 1µl of prepared ladder was pipetted in well maked with ladder before pipetting 1 µl of sample in each of the 12 sample wells. Then the chip was put horizontally in the adapter of the IKA MS3 Vortexes for 1 minute at 1200g prior to run the chip in the Agilent 2100 bioanalyzer. After getting data, the chip was discarded and electrode was clean by using 350ml of Diethylpyrocarbonate (DEPC) and RNase away water to avoid further contamination. Precaution was taken to avoid light exposure in gel-dye by covering aluminum foil paper and ice-flakes were used to maintain the temperature in all steps.

3.5. Comparison of TaqMan and Affinity Script kit

Reverse transcription is a process by which single-stranded RNA is reverse transcribed into complementary DNA (cDNA) by using a reverse transcriptase enzyme (RT), a primer, dNTPs and an RNAase inhibitor. TaqMan and Affinity Super Script kits were compared for selecting better kit for cDNA synthesis. In addition, two different RNA amount (200ng RNA and 470ng RNA) were also compared using two kits separately.

All steps of the procedure were performed at room temperature. During the procedure, is necessary to work quikly. All centrifugation steps were performed at 20-25°C in a standard microcentrifuge with proper caution that the centrifuge does not cool below 20°C.

TaqMan kit

The TaqMan Gold RT-PCR Kit was designed for the reverse transcription (RT) and polymerase chain reaction (PCR) amplification of a specific target RNA from either total RNA or mRNA. The reagent in this kit was used for one-step or two-step RT-PCR with ABI PRISM® Sequence Detection Systems.

Oligo-dT primer was used in the synthesis of cDNA with the TaqMan Gold RT-PCR kit and five reactions with two different samples were prepared according to the recipe in Table 3.2.

Table 3.2. TaqMan RT-PCR protocol

Reagent	Volume (µg)	Reaction times	Total volume (µg)
10×RT buffer	2.5	5	12.5
25mM MgCl2	5.5	5	27.5
2.5Mm dNTp mixture	5	5	25.0
Oligo dT	1,25	5	6.25
MultiScribe RT enzyme	1,56	5	7.8
RNase Inhibitor	0.5	5	2.5

RNase free water mixture was used to fill up the RNA solution to 8.7 µl

Affinity Script

The kit combines the high-performance of Affinity Script RT with Herculase II fusion technology, strand master mix and oligo-dT and five reactions with two different samples for following protocol.

Table 3.3. Affinity Script RT-PCR protocol

Reagent	Volume (µg)	Reaction times	Total volume (µg)
Strand Master Mix (2X)	10.0	5	50
Oligo dT	3	5	15
Affinity Script (RT) RNA block	1.00	5	5

RNase free water mixture was used to fill up the RNA solution to 6.00 µl

The PCR programme was

25°C for 10 minutes (primer incubation)

45°C for 60 minutes (RT step)

95°C for 5 minutes (RT inactivation)

4°C for infinite

All RT products (cDNAs) were stored at -20°C

3.6. cDNA synthesis protocols

cDNA synthesis protocol B was followed from two different protocols (Protocol A and B). Protocol A without RT and B without RT were used to assess genomic DNA contamination.

Protocol B & No RT

Concentration of total RNA 210.91 ng/µL

Table 3.4. PCR protocol B

Reagent	Volume (µl)	Reaction times	Total volume (µl)
Strand Master Mix (2X)	10.0	53	530
Oligo dT	1.7	53	90.1
Affinity Script (RT) RNA block	1.00	53	53
Random Hexamers/primers	0.3	53	15.9
Total	13		

Volume of 700 ng RNA sample was 3.32 µL (700/210.91).

RNase free water mixture was:

RNA volume 3.32+2.68 (H_2O) = 6.0

No RT sample with 3. 68 H_20

The mixture was kept on ice

The PCR programme was

25°C for 5 minutes (primer incubation)

42°C for 45 minutes (RT step)

95°C for 5 minutes (RT inactivation)

4°C for infinite

Protocol A & no RT

Oligo dT + RH & high RNA input and high Temperature

Table 3.5. PCR protocol A

Reagent	Volume (µl)	Reaction times	Total volume (µl)
Strand Master Mix (2X)	10.0	53	530
Oligo dT	3	53	159
Affinity Script (RT) RNA block	1.00	53	53
Total	14		

Volume of 1400ng RNA sample was 6.64 µL (1400/210.91)

RNase free water mixture was:

RNA volume 6.64+0.36 (H_2O) = 7.0

No RT tube: 1.36 H_20

The mixture was kept on ice.

The PCR programme was

25°C for 5 minutes (primer incubation)

42°C for 5 minutes (RT step)

55°C for 15 minutes (RT step)

95°C for 5 minutes (RT inactivation)

4°C for infinite

The RT products (cDNAs) were stored at -20°C

3.7. Real-time qPCR

Expressions of 19 genes are analyzed by real-time qPCR. The PCR primers (Table 6) are designed using the Vector NTI (Invitrogen, Carlsbad, CA, USA) and synthesized by Invitrogen. Efficiency is checked from tenfold serial dilutions of cDNA for each primer pair. Syber® Green (6.0 µl), primer (F/R: 1/1 µl) and the cDNA (4.0 µl) template are mixed in 12 µl volumes for real-time qPCR. PCR is performed in duplicates in 96-well optical plates on Light Cycler 480. The specificity of PCR amplification is confirmed by melting curve analysis. Relative expression of mRNAis calculated using the -ΔΔCt method. Elongation factor 1A (EF1A) is chosen as reference gene which was tested by Todorcevic et al., (2010a) using the BestKeeper and GeNorm software. Differences between control and treatments are assessed with Student's t-test ($p < 0.05$).

Table 3.6. Real-time qPCR primers

TARGETS: gene names and symbols	PRIMERS: forward and reverse (5′-3′)	GenBank accessions
CCAAT/enhancer-binding protein alpha (C/EBPα)	F: ATGGAGCAACCAAATCTCTATG R: CTGGCAGTTGGCCATGGG	EU668995
Fatty acid synthase (FAS)	F: TGCCTCAGCACCCTACT R: GCTTTACAACCTCAGGA	BT048827
17β-hydroxysteroid dehydrogenase type IV (HSD17B4)	F: GGATCGTTCCTTTGGCAGAAC R: TGGTTCCAGGCAGCTCTAGTGAC	CA370123
Peroxisome proliferator activated receptor-γ coactivator-1 alpha (PGC-1a),	F:GTCAATATGGCAACGAGGCTTC R:TCGAATGAAGGCAATCCGTC	FJ710605
UDP-glucuronosyltransferase (UGT)	F: GGTGGCGATGTCCACTATTT R: GTCCAGGAGGCCATCTATCA	CA342060
Glutathione peroxidase-2 (GPx-2)	F: GTACCTCAAGGAGAAGCTGCCGT R: ATTAAGGCCATGGGATCGTCGC	CF753103
Tumor necrosis factor-alpha (TNF-α)	F: GCTTGTCTCTTGTTGCCACCA R: TGTGTGGGATGAGGATTTGGTT	AF321836
Signal transducers and activators of transcription 1(STAT1)	F:GAACATGGAGGAGTCCAATGGAAGC R: GGACCCTCATTTGATCTGTTGCCT	CA343225
Leukocyte cell-derived chemotaxin 2 (LECT2)	F: CTGTGTTGTCAGAGTGCGAGATGGT R: TACACACAATGTCCAGGCCCTGA	EXOB2G01
Interleukin 15 (IL-15)	F: TTGGTTTTTGCCCTAACTGC R: CAGGTCCATCGCACTCTTTT	S35506545
Interleukin-1 beta (IL-1β)	F: GTATCCCATCACCCCATCAC R: TTGAGCAGGTCCTTGTCCTT	CA377361
Interleukin-13 receptor subunit alpha-2 (IL-13Rα2)	F: TCCCTGTGGTCCTCAACTTC R: GCCTGGATCTCCTCATCATC	CA048395
Arachidonate 5-lipoxygenase (ALOX5)	F: TCTGAGTCATGCTGTCCGTAGTGGT R: CCTCCCTCTCTACCTTCGTTGCAAA	CA387866
Arachidonate 5-lipoxygenase-activating protein (ALOX5AP)	F: TCTGAGTCATGCTGTCCGTAGTGGT R: CCTCCCTCTCTACCTTCGTTGCAAA	CA369467
TNF decoy receptor (RTEL1)	F: TCTCCTGGTATTTGCGCTCTGTGGT R:TATAAGTCGGTGTGTGAGCGCCTGA	CA351440
Interleukin-1 receptor antagonist 1 (IL-1RA)	F: TCTCTGAGCCGCTCAACCTGTCAT R: CGTTCCACGACAGCTTTATACGGA	CA348044
Transforming growth factor beta (TGF-β)	F: AATCGGAGAGTTGCTGTGTGCGA R: GGGTTGTGGTGCTTATACAGAGCCA	EU082211
Interleukin-6 (IL-6)	F: ATGCTCGTCCACGAGGTAACC R: TACCTCAGCAACCTTCATCTGG	CA377360

CHPTER IV

RESULTS

The cDNA from Atlantic salmon differentiating adipocytes are analysed with qPCR. All treatment groups are cultivated in the same media, with two different concentrations of omega-3 fatty acids (FAs) (0.3 and .05 mM) and in addition with either vitamin E (0.1 mM), PGN (10 mM) or vitamin E+PGN. qPCR is a method of choice for more detailed investigation on individual aspect of expression levels. We examined the expression level of adipogenic (C/EBPα), fatty acid and energy metabolism (FAS, HSD17B4, PGC-1α, UGT), intracellular antioxidant (GPx-2), immune; pro-inflammatory (TNF-α, STAT1, LECT2, IL-15, IL-1β, IL13a2, ALOX5 and ALOX5AP), anti-inflammatory (RTEL1, IL-1RA, IL-10 and TGF-β) related markers to understand the effect of different does of omega-3 FAs.

4.1. Effects of different doses of omega-3 FAs, vitamin E and immunostimulant (peptidoglycan) on Adipogenesis

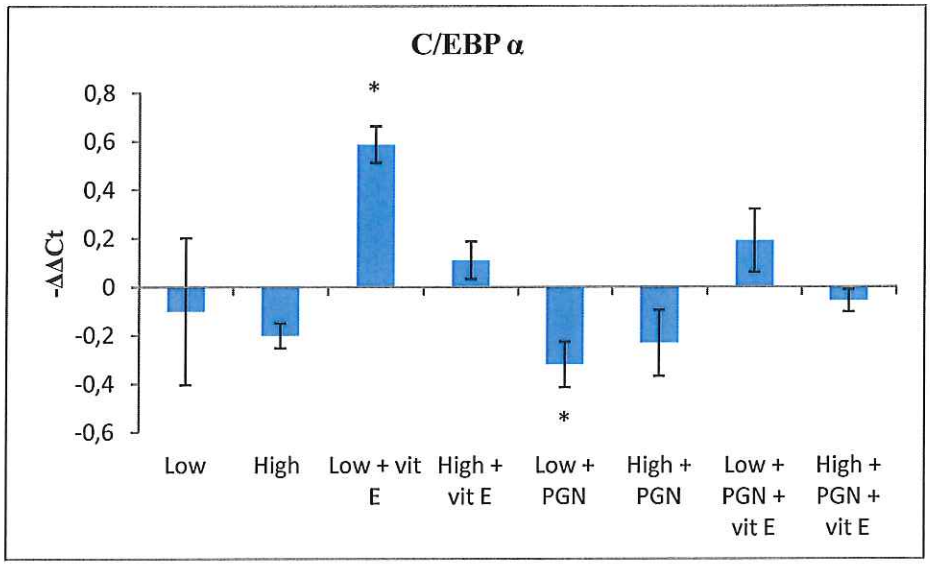

Figure 4.1: Adipogenic transcription factor analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Significance difference from control is indicated with a star (*). Control= all pooled treatment groups.

CCAAT/enhancer-binding protein alpha (C/EBPα) regulates Adipogenesis by turning on fat-specific genes that are necessary for the synthesis, uptake and storage of long chain fatty acids (Dalen et al., 2004). This transcription factor is expressed realtively late during

adipogenesis. However, the opposite effects of vitamin E and PGN were seen from above figure; while vitamin E increases the expression of C/EBPα, PGN lowers it. Moreover, under the high omega-3 FAs doses changes were not as dramatic.

4.2. Effects of different doses of omega-3 FAs, vitamin E and immunostimulant (peptidoglycan) on lipogenesis and energy storage

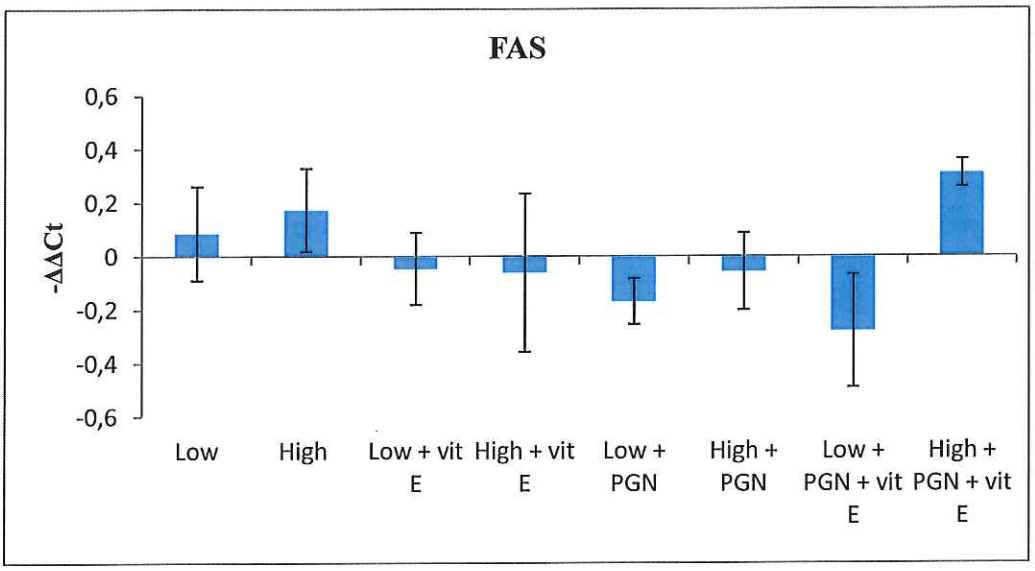

Figure 4.2: Fatty acid and energy metabolism related marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Control= all pooled treatment groups.

Fatty acid synthase (FAS) is a major lipogenic enzyme catalyzing the synthesis of long-chain saturated fatty acids (Wakil, 1989). Expression of FAS is linked with storage of energy in liver and in adipose tissue. Decrease activities and gene expression of lipogenic enzymes, are evidenced by the decrease in FAS mRNA expression (Huang et al., 2007).

The treatment with high omega-3 FAs influences FAS induction and PGN stimulates down regualtion in both low and high omega-3 FAs treatment. However, inductions of FAS with low and high omega-3 FAs are negatively influenced by vitamin E.

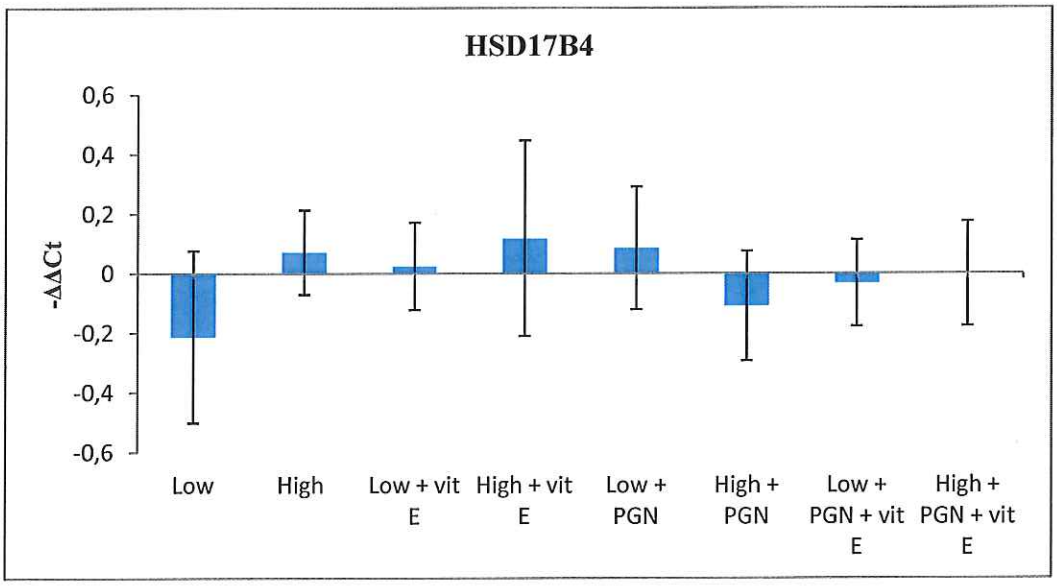

Figure 4.3 : Fatty acid and energy metabolism related marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Control= all pooled treatment groups.

17β-hydroxysteroid dehydrogenase type 4 (HSD17B4) that involve in catalysis of the steroid molecules in androgen and estrogen biosynthesis thereby modulating the biological function of the steroid hormones. 17β-HSD4 is involved in the inactivation of steroids. In addition, HSD17B4 has involvement in β-oxidation of very long chain fatty acids (Suzuki et al., 1997). The most important biological functions of 17β-HSD4 *in vivo* are in the metabolism of fatty acids and sterols (Breitling et al., 2001). 17β-HSD4 is induced with high omega-3 FAs treatment where low level of omega-3 FAs shows negative induction. However, under high omega-3 FAs does' vitamin E shows increased level of induction with higher variability. Moreover, PGN influences to decrease in same treatment. Moreover, vitamin E with low omega-3 FAs treatment promotes expression of 17β-HSD4 but in combination with PGN appears to decrease level of induction.

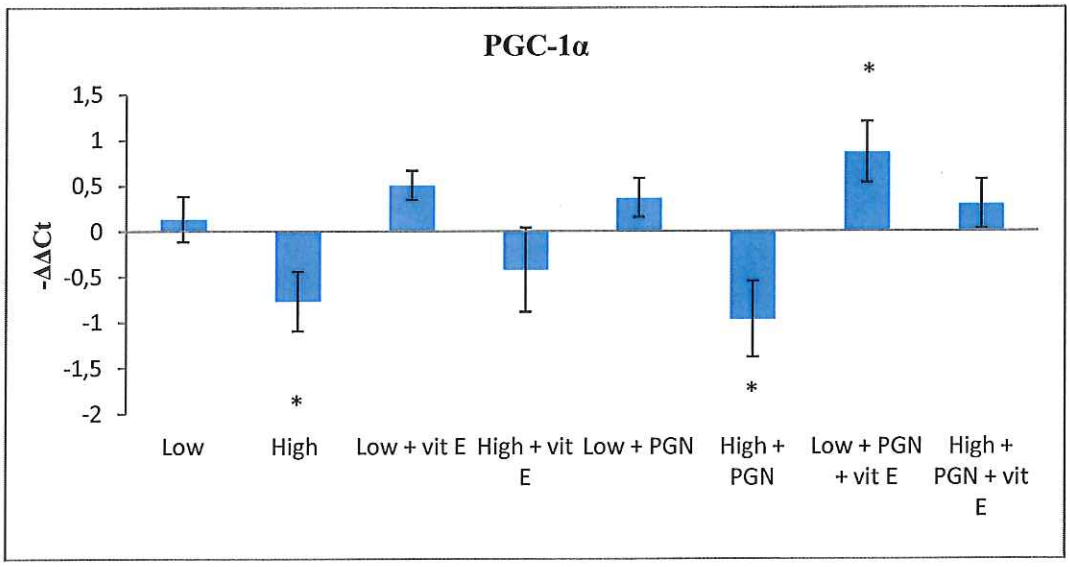

Figure 4.4: Fatty acid and energy metabolism related marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Significance difference from control is indicated with a star (*). Control= all pooled treatment groups.

Peroxisome proliferator activated receptor-γ coactivator-1 alpha (PGC-1α), is a transcriptional coactivator that regulates the genes involved in energy metabolism that activates mitochondrial biogenesis, oxidative metabolism and fatty acid β-oxidation with the final aim of providing a more efficient pathway for aerobic energy production. This protein interacts with the nuclear receptor PPAR-γ, which permits the interaction of this protein with multiple transcription factors. PGC-1α is mainly expressed in tissues with high energy oxidative capacity adipose tissue, and is robustly induced in conditions that require energy, such as cold, fasting and exercise (Puigserver et al., 1998). However, under high omega-3 FAs treatments show significant induction of PGC-1α. In addition, PGN reveals down regulating tendency with high omega-3 FAs treatments. Moreover, level of PGC-1α expression is promoted with Low+vitamin E treatments. Nonetheless, Low+PGN+Vitamin E treatments reveal the significant highest induction.

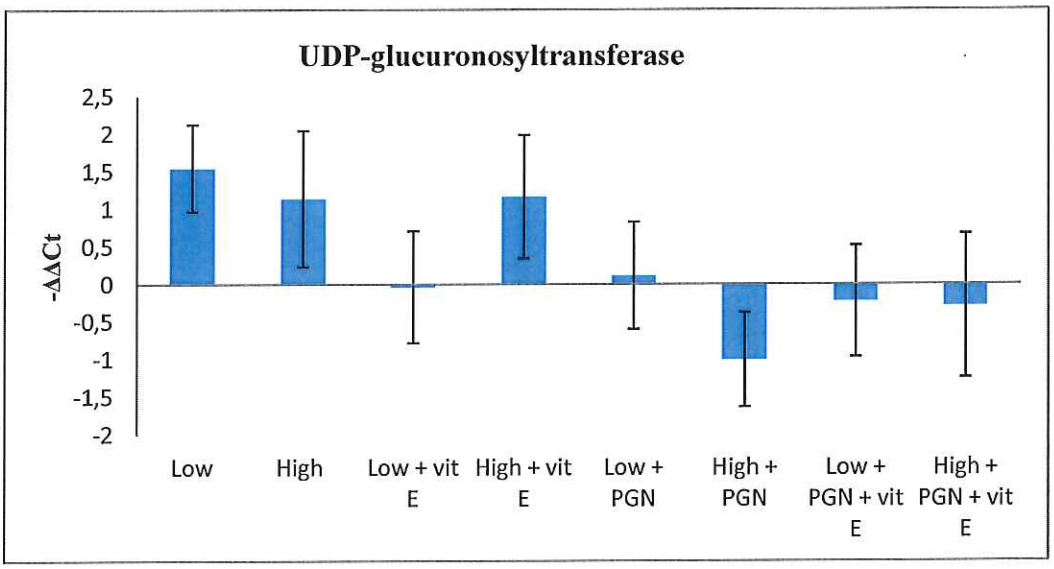

Figure 4.5: Fatty acid and energy metabolism related marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Control= all pooled treatment groups.

UDP-glucuronosyltransferase (UGT) enzyme is key proteins that catalyze the glucuronidation reaction on a wide range of structurally diverse endogenous and exogenous chemicals. Glucuronidation is one of the major phase II drug-metabolizing reactions that contribute to drug biotransformation. This biochemical process is also involved in the homeostasis of numerous endogenous molecules.

UGT is more positively influenced with low omega-3 FAs than high omega-3 FAs treatments. However, in combination with vitamin E and low omega-3 FAs shows reduced expression with high variability. The effect of vitamin E is almost unchanged with high omega-3 FAs treatments. On the contrary, PGN shows more negative effects with high omega-3 FAs treatments than with low omega-3 FAs doses.

4.3. Intracellular antioxidant marker

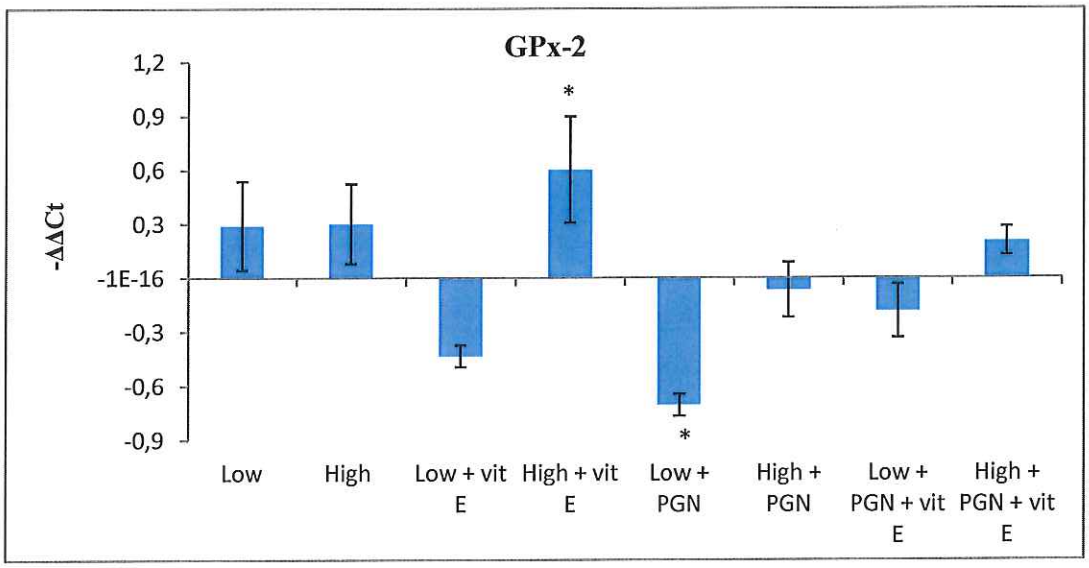

Figure 4.6: Lipogenic transcription factor analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Significance difference from control is indicated with a star (*). Control= all pooled treatment groups.

Glutathione peroxidase -2 (GPx-2) is responsible for protecting cells from damage due to free radicals like hydrogen and lipid peroxides. Triacylglyceride (TAG) deposition requires the inhibition of reactive oxygen species (ROS). Vitamin E shows both antioxidant (Burton et al., 1998) and non-antioxidant (Boscoboinik et al., 1991) properties in expression of GPx-2 in high (up-regualtion) and low (down-regualtion) omega-3 FAs treatments respectively. However, vitamin E and PGN shows the opposite effect on adipose cells; GPx-2 was up-regulated in high+vitamin E while its expression significantly dropped in low+PGN group.

4.4. Immune related marker

4.4.1. Effect of omega-3 FAs, vitamin E and immunostimulant (peptidoglycan) to pro-inflammatory responses

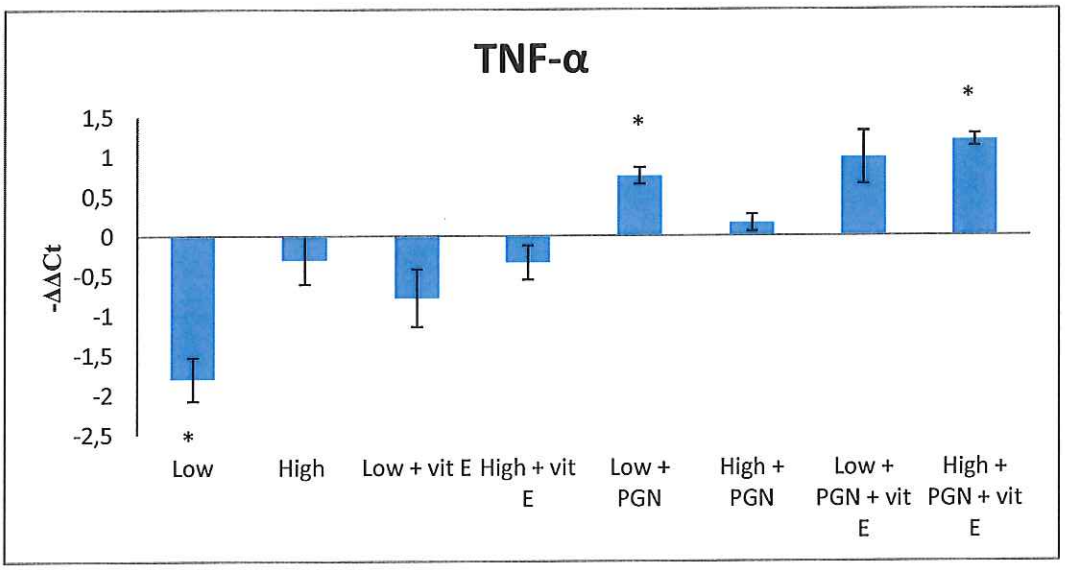

Figure 4.7: Pro-inflamatory marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt±SE (n=5). The significance level has been set at $p \leq 0.05$. Significance difference from control is indicated with a star (*). Control= all pooled treatment groups.

Tumor necrosis factor-alpha (TNF-α), is a pleiotropic inflammatory cytokine involved in systemic inflammation and is a member of a group of cytokines that participates in both inflammatory disorders of inflammatory and non-inflammatory origin. This cytokine possesses both growth stimulating properties and growth inhibitory processes, and it appears to have self regulatory properties as well. TNF-α increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection. Lipopolysaccharide from from bacteria cell walls is an especially potent stimulus for TNF-α synthesis.

TNF- α is induced significantly less with low treatment of omega-3 FAs than high does. While low+vitamin E helps to come down the level of TNF- α. However, high+vitamin E does not show any remarkable changes in context with high omega-3 FAs treatment. In addition, PGN significantly influences to induce TNF- α in adipocytes.

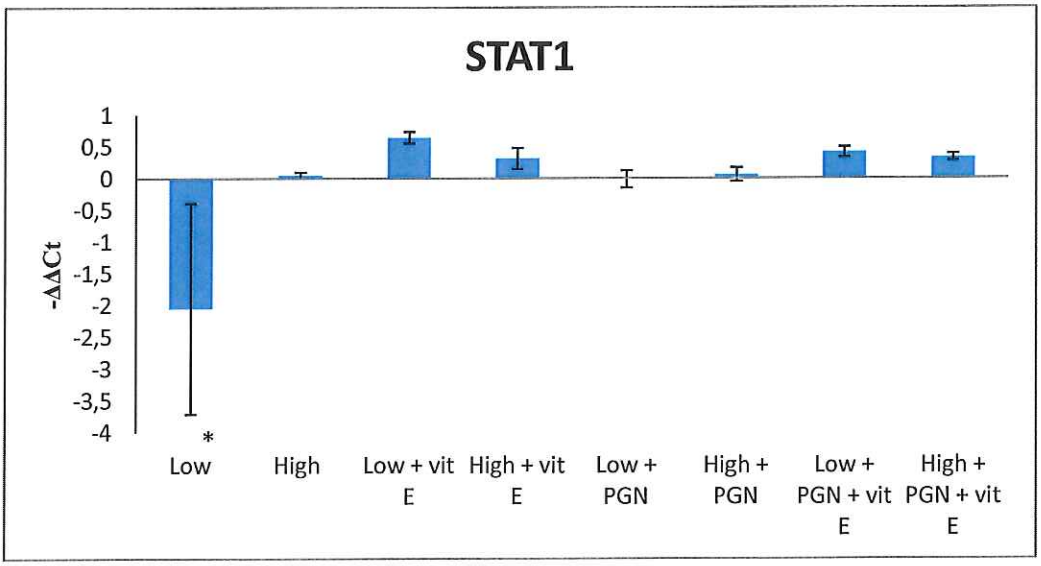

Figure 4.8: Pro-inflamatory marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Significance difference from control is indicated with a star (*). Control= all pooled treatment groups.

STAT1 (signal transducers and activators of transcription 1) protein encoded by the gene is a member of the STAT protein family. In response to cytokines and growth factors, STAT 1 is phosphorylated by the receptor associated kinases, and act as transcription activators.

STAT1 is significantly down regulated with low omega-3 FAs treatments. The effect of vitamin E is appeared in both treatments of low and high omega-3 FAs by induction.

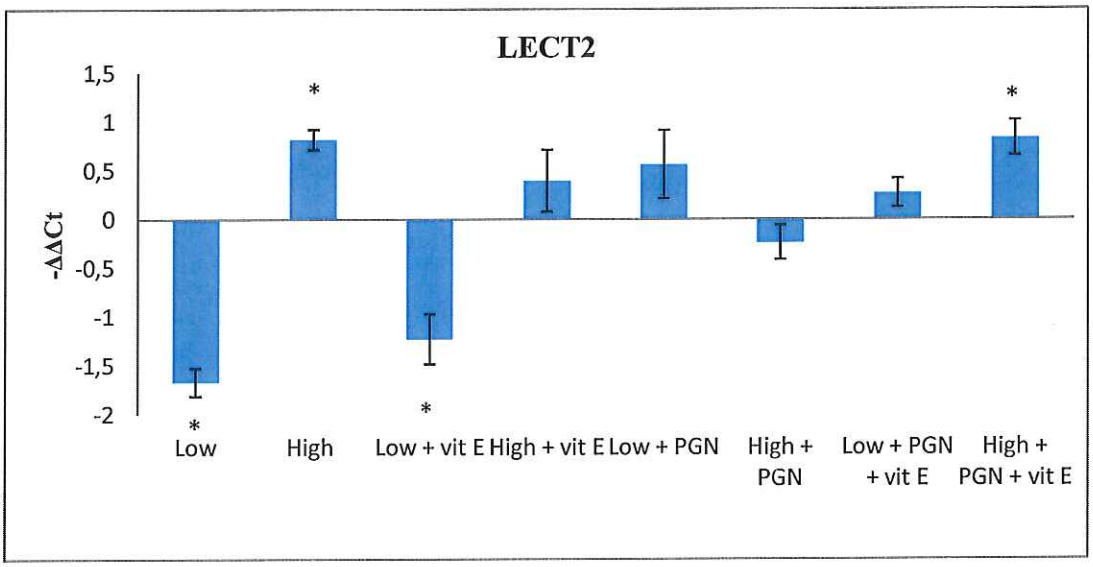

Figure 4.9: Pro-inflamatory marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Significance difference from control is indicated with a star (*). Control= all pooled treatment groups.

Leukocyte cell-derived chemotaxin 2 (LECT2), that acts as a multifunctional protein that involved in cell growth, differentiation and autoimmunity. LECT2 is a cytokine which is sensitive to bacterial infections (Lin et al., 2007) and LPS treatment in fish (Djordjevic, 2009) in immune tissue. Dose response effects have been seen transparently from low and high treatments in LECT2 expression. However, high omega-3 FAs show LECT2 expression which is come down by vitamin E. Under low omega-3 FAs, vitamin E influences to increase the induction levels. However, PGN influences to increase and decrease the level of expression in low and high omega-3 FAs treatments respectively.

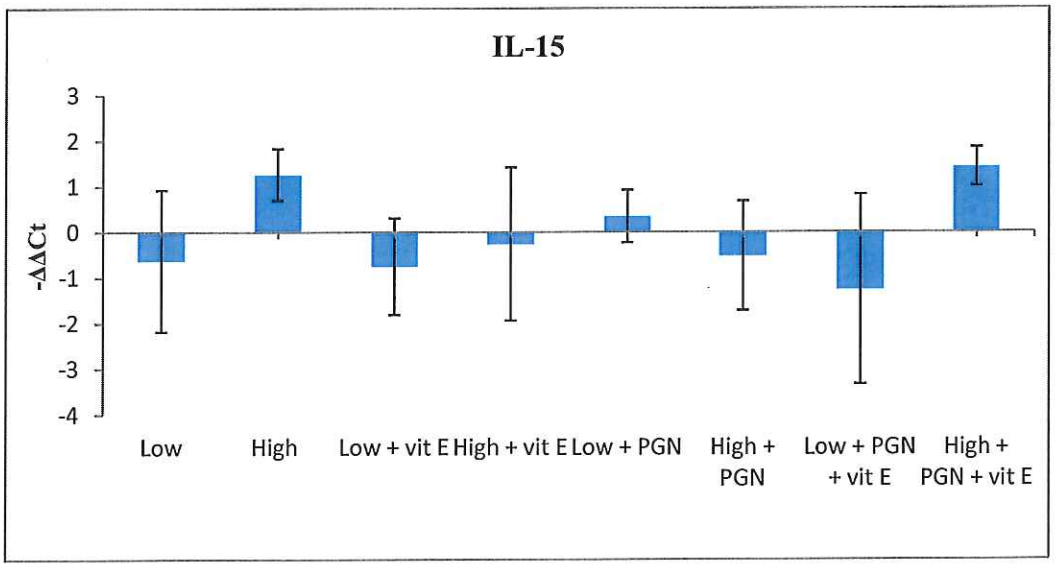

Figure 4.10 : Pro-inflamatory marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Control= all pooled treatment groups.

Interleukin 15 (IL-15) is a pro-inflammatory cytokine that plays role in T and natural killer (NK) cell activation and proliferation. This cytokine is also implicated in NK cell development.

IL-15 to normal rats caused significant reductions in white adipose tissue WAT mass via both a decreased rate of tissue lipogenesis and a reduction in lipoprotein lipase (LPL) activity, without concomitant reductions in food intake (Belén et al., 2011). Inappropriate expression of IL-15 has also been linked with a number of immune and inflammatory mediated diseases (Waldman and Hubbard, 2006). However, high range of standard error reveals high variability in between samples. There is no significant difference seen from above figure. Under low omega-3 FAs treatment vitamin E appears to decrease IL-15 expression, and PGN shows tendency to up-regulate the level of induction. The effect of high omega-3 FAs by induction of IL-15 is affected by PGN.

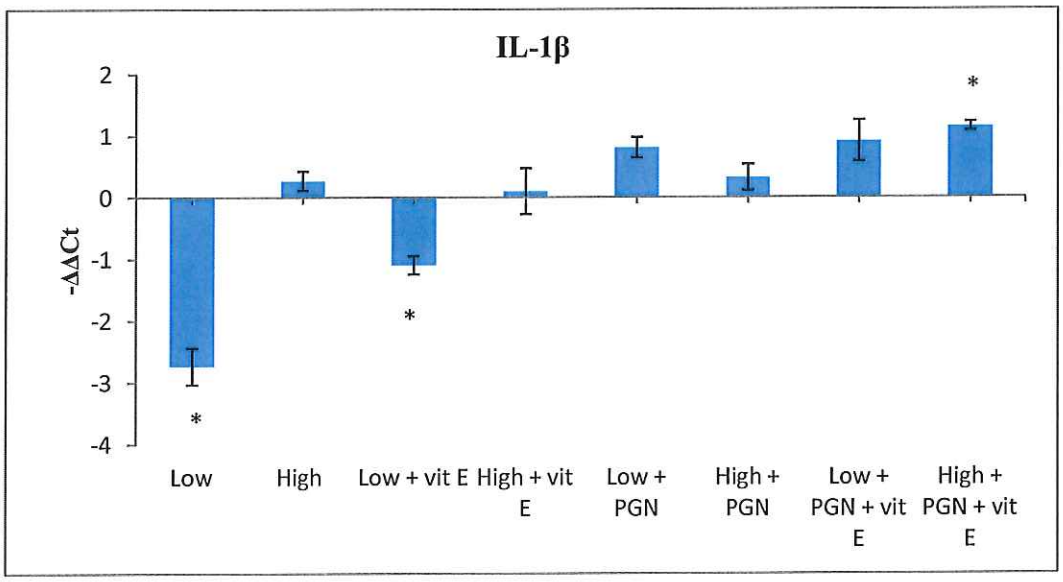

Figure 4.11: Pro-inflamatory marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Significance difference from control is indicated with a star (*). Control= all pooled treatment groups.

Interleukin-1 beta (IL-1β) also known as pro-inflammatory cytokine produced by activated macrophages as a proprotein which plays a key role in the induction of the complex immune response. IL-1β also exerted negative effects on cellular insulin content and DHA (Aarnes et al., 2002). In mammals, IL-1β is produced in response to many stimuli which include bacterial LPS, numerous microbial products, cytokines (Stylianou, 1992). IL-1 β was down-regulated in Low and Low+vitamin E groups while PGN had a effect under the combination with High+PGN+Vitamin E (highest up-regulation). The effect of omega-3 FAs in context of expression is positive by high omega-3 FAs treatments. However, vitamin E help to up-regulate the expression of IL-1β. Nonetheless, low omega-3 FAs does influences to decrease expression of IL-1b. The effects of omega-3 FAs with PGN treatments are crystal enough where IL-1β is induced comparatively higher by low omega-3 FAs.

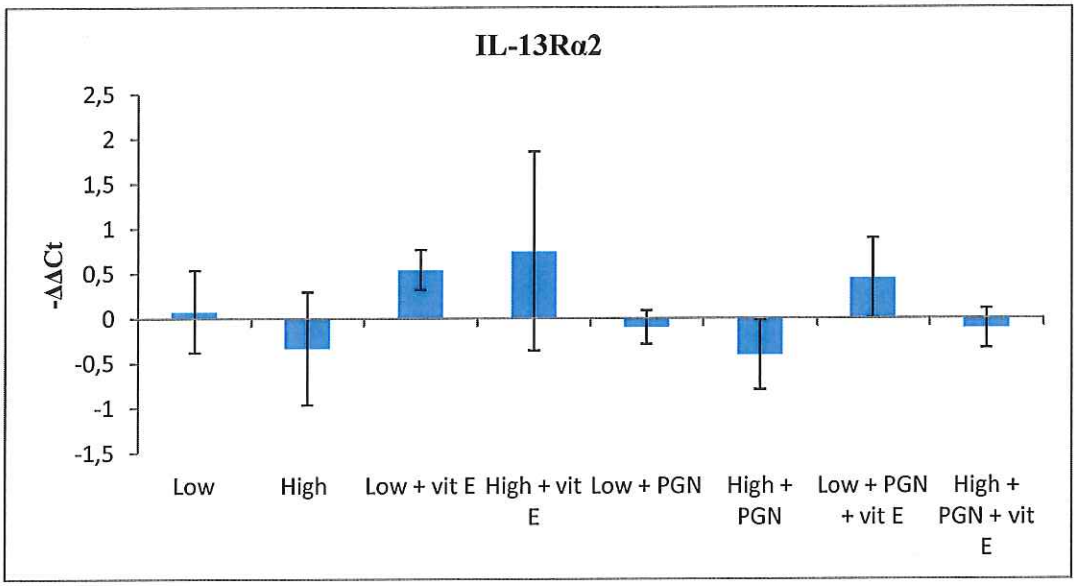

Figure 4.12: Pro-inflamatory marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Control= all pooled treatment groups.

Interleukin-13 receptor subunit alpha-2 (IL-13Rα2) which is closely related to IL-13Rα1, a subuint of the interleukin-13 receptor complex. It is, however able to regulate the effects of both IL-13 and IL-4.

IL-13Rα2 is significantly down regulated by vitamin E treatment with WAT of Salmon (Todorcevic et al., 2010a) but presented graph shows induction of IL-13Rα2 in combination of low omega-3 FAs and vitamin E treatment. Low and high omega-3 FAs doses show an opposite induction trend. However, vitamin E helps to increase the level of expression with both low and high omega-3 FAs treatments. Moreover, IL-13Rα2 expression is positively influenced by low omega-3 FAs. Nonetheless, PGN shows inhibition of IL-13Rα2 expression.

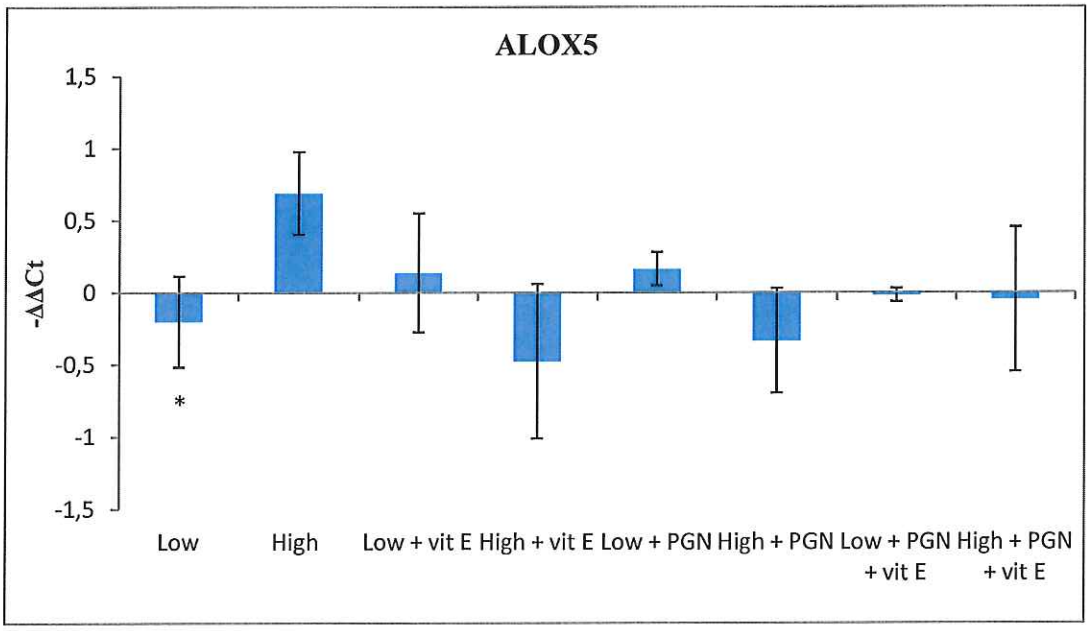

Figure 4.13 : Pro-inflamatory marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Significance difference from control is indicated with a star (*). Control= all pooled treatment groups.

ALOX5 encodes arachidonate 5-lipoxygenase, a cystolic enzyme that catalyzes the two-step conversion of arachidonic acid to leukotriene. Leukotrines promote inflammation by attracting neurtophils and increasing vascular permeability. Low does of omega-3 FAs show negative regulation while vitamin E helps to increase the level of induction. On the contrary, an opposite figure is seen from high omega-3 FAs and high +vitamin E treatments. Besides, high variability is seen from high+PGN+vitamin E treatments.

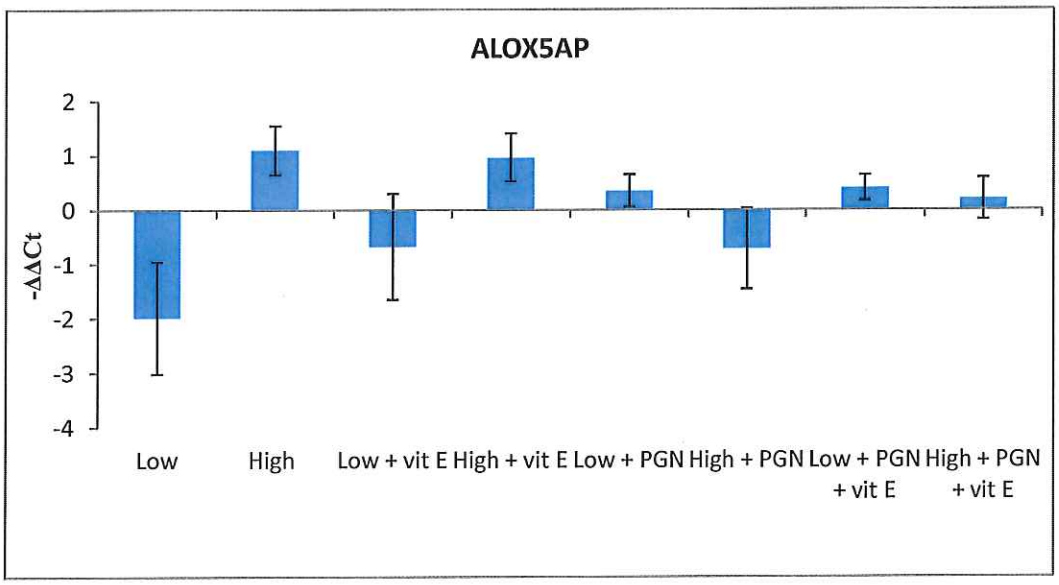

Figure 4.14: Pro-inflamatory marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Control= all pooled treatment groups.

ALOX5AP/FLAP (Arachidonate 5-lipoxygenase-activating protein), is required for leukoritene synthesis. ALOX5AP inhibit of its function impede translocation of 5-lipoxygenase from the cytoplasm to the cell membrane and inhibit 5-lipoxygenase activation. ALOX5AP is expressed with high does of omega-3 FAs and in combination with vitamin E which is affected (down-regulation) by PGN. On the contrary, a reciprocal figure is seen for all low omega-3 FAs treatments.

4.4.2. Effect of omega-3 FAs, vitamin E and immunostimulant (peptidoglycan) to anti-inflammatory responses

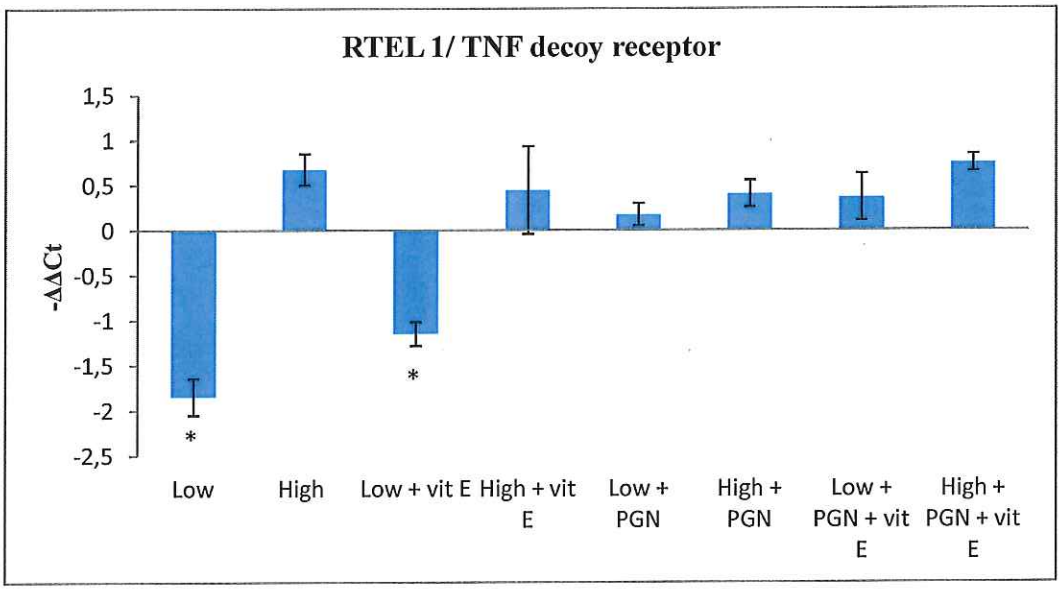

Figure 4.15: Anti-inflammatory marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Significance difference from control is indicated with a star (*). Control= all pooled treatment groups.

TNF decoy receptor plays a vital role to inhibit apoptosis mediated by pro-apoptotic TNF family of cytokines which shows in some teleost fishes. It also reveals crucial roles in both innate and adaptive immunity, such as regulation of cell proliferation, differentiation, cell survival, cell death, cytokine production, and lymphocyte co-stimulation. RTEL1, decoy receptor antagonist of TNF-α that prevents damage from excessive activity of cytokine. The significant differences are seen from low omega-3 FAs and Low +vitamin E treatments. However, high omega-3 fatty acids' dose with all treatments shows induction of TNF decoy receptor.

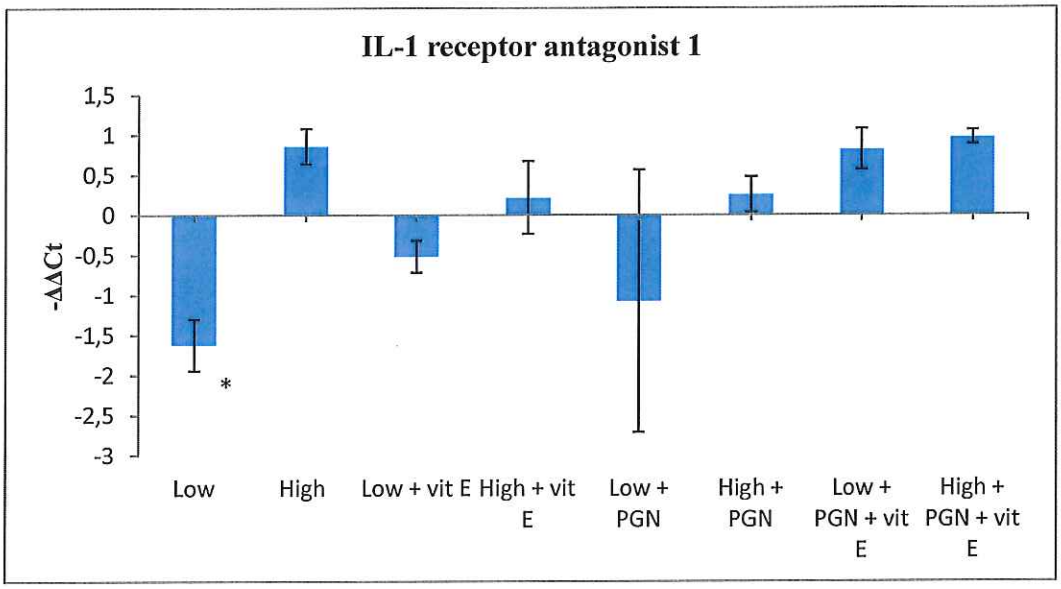

Figure 4.16: Anti-inflammatory marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Control= all pooled treatment groups.

The interleukin-1 receptor antagonist 1 (IL-1RA) is a cytokine family protein that inhibits the activities of IL-1 related immune and inflammatory responses. Immune cells (Roux-Lombard et al., 1989) and adipocytes (Juge-Aubry et al., 2003) cells are known to secrete IL-1RA. Low doses of omega-3 FAs influence down-regulation of IL-1RA. However, under high omega-3 FAs treatments, IL-1RAis induced. In addition, the level of induction with high omega-3 FAs is affected by vitamin E. Vitamin E shows increased levels of expression in both low and high omega-3 FAs with PGN treatments. Besides, high variability of expression level is seen from Low+PGN treatments.

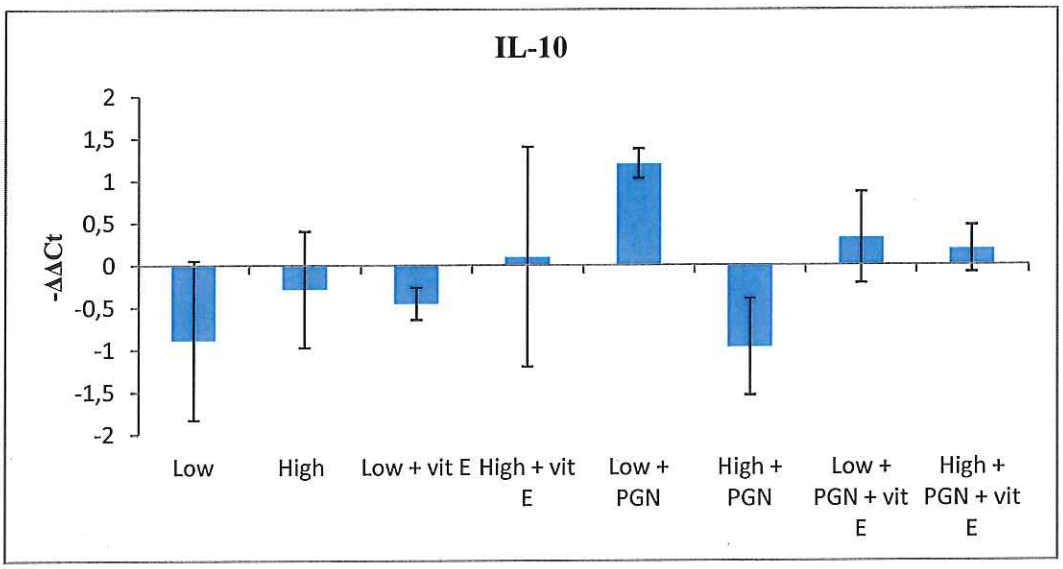

Figure 4.17: Anti-inflammatory marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Control= all pooled treatment groups.

Interleukin-10 (IL-10) is an anti-inflammatory cytokine produced by a range of cell types including macrophages, monocytes, T and B lymphocytes, keratinocytes and tumor cells (Goldman and Velu, 1995). IL-10 is more negatively influenced with low omega-3 FAs than high omega-3 FAs treatments. In combination with vitamin E and high omega-3 FAs, shows high variable induction. However, vitamin E helps to increase level of induction in combination with low omega-3 FAs.

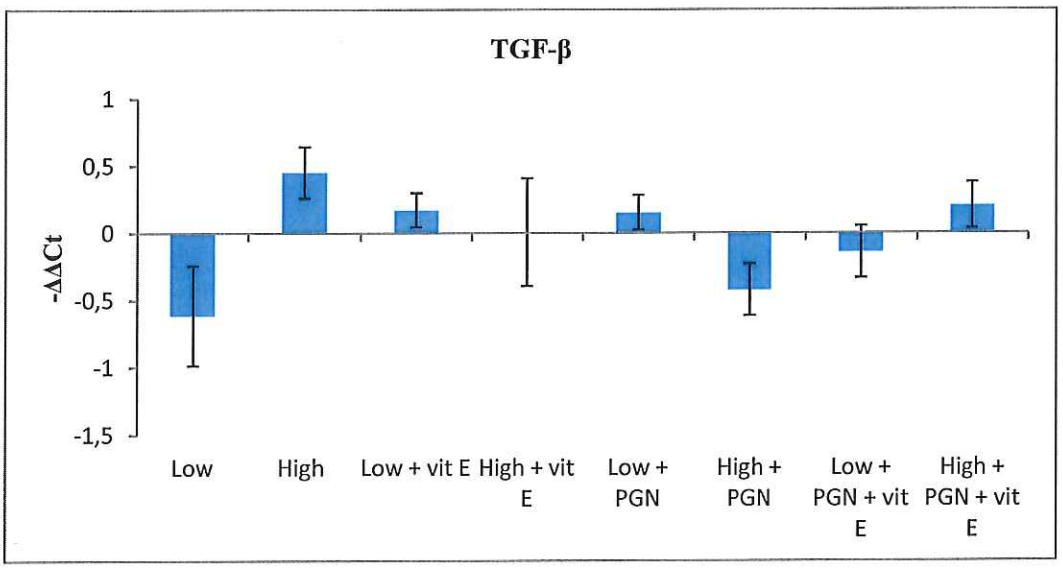

Figure 4.18: Anti-inflammatory marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Control= all pooled treatment groups.

Transforming growth factor beta (TGF-β) is believed to be important in regulation of the immune system. TGF-β inhibits adipocyte differentiation by repressing C/EBP transactivation function (Choy and Derynck, 1998). TGF-β is induced under the treatment of high omega-3 FAs. However, vitamin E influences induction of TGF-β in Low+vitamin E treatment. On the contrary, high variability is showed from High+vitamin E treatments. However, low omega-3 FAs with PGN shows reciprocal induction in comparison with High+PGN treatment.

4.5. Pro/anti-inflammatory marker

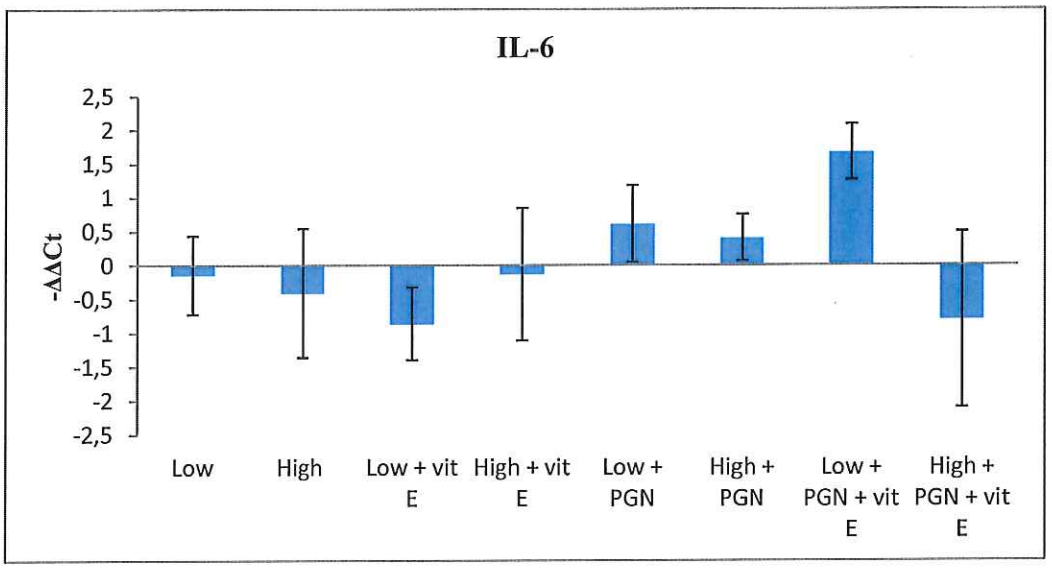

Figure 4.19: Pro/anti-inflammatory marker analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Control= all pooled treatment groups.

Interleukin-6 is considered the major inflammatory mediator in obesity. It implicates in the release of triglycerides and free fatty acids, down regulated lipoprotein lipase, increased production of ROS (Eder et al., 2009). Vitamin E indicates inhibitory role to up-regulate IL-6 where PGN stimulates induction. The comparative level of IL-6 expression appears to higher with low omega-3 FAs than high omega-3 FAs. However, vitamin E in combination with low omega-3 FAs shows comparatively higher negative regulation than High+vitamin E. In addition, PGN reveals more positive regulatory flows with low omega-3 FAs treatments. However, under combined effects of Low+PGN+vitamin E shows the highest level of induction.

4.6. Comparative genes expression (pro-/pro-, anti-/anti- and pro-and anti-inflammatory genes)

Comparative gene expression profiles have been analyzed to figure out an overview of combined effects of different pro-and anti-inflammatory genes. Anti-/anti- (TGF-β: IL-10), pro-/anti- (IL-1β:IL-1RAand TNFα:RTEL1) and pro-/pro-inflammatory (ALOX5: ALOX5AP) genes are presented to achieve better interpretation of results.

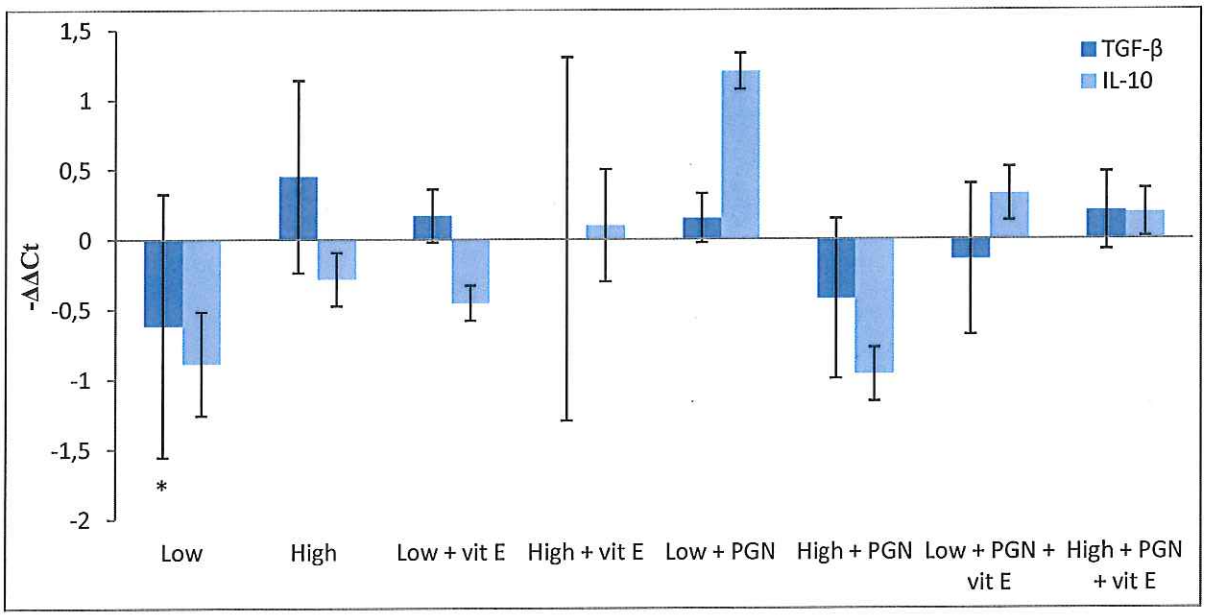

Figure 4.20: Comparision between anti-inflammatory cytokines analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Significance difference from control is indicated with a star (*). Control= all pooled treatment groups.

TGF-β and IL-10 are both anti-inflammatory cytokines. PGN is the strongest inducer of IL-10 under low omega-3 FAs treatment. IL-10 is needed to counteract the PGN-induced inflammation in developing adipocytes. Low expression of anti-inflammatory cytokines TGF- β and IL-10 under PGN stimulation at high omega-3 FAs treatment is most probably the consequence of the lower induction of inflammation in cells exposed to higher doses of EPA and DHA > (Fig. 17&18) High+ PGN in other analysed immune-related genes, gene pairs above and a summary of pro-inflammatory genes in the figure below.

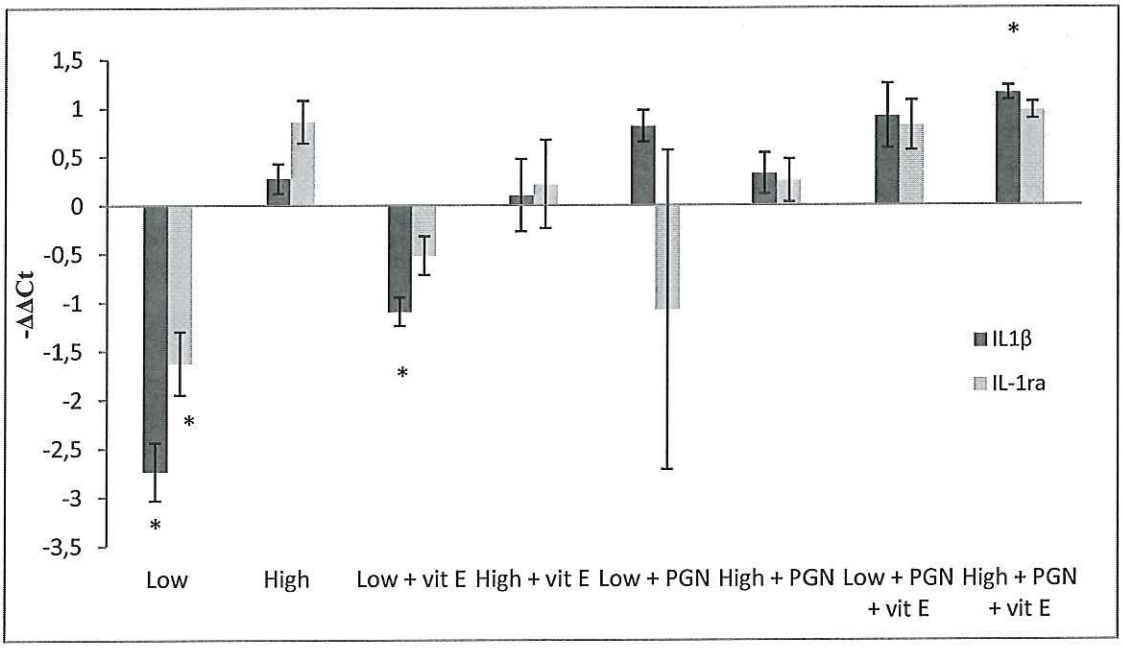

Figure 4.21. Comparision between pro-inflammatory cytokine and its antagonist, analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Significance difference from control is indicated with a star (*). Control= all pooled treatment groups.

IL-1β is a pro-inflammatory cytokine implied in inflammatory responses but also, as recently shown, in the regulation of energy metabolism. IL-1ra is the antagonist of IL-1β. Difference between Low+PGN and High+PGN is important: the magnitude of difference between IL-1β and IL-1RAis noteworthy in Low+PGN where IL-1β acts as antagonist of IL-1ra.

Anti-inflammatory effect of vitamin E is also remarkable, especially under the combined low omega-3 FAs and PGN treatment where it manages to substantially increase the expression of anti-inflammatory IL-1ra (roughly 4 fold in comparison to the Low+PGN group).

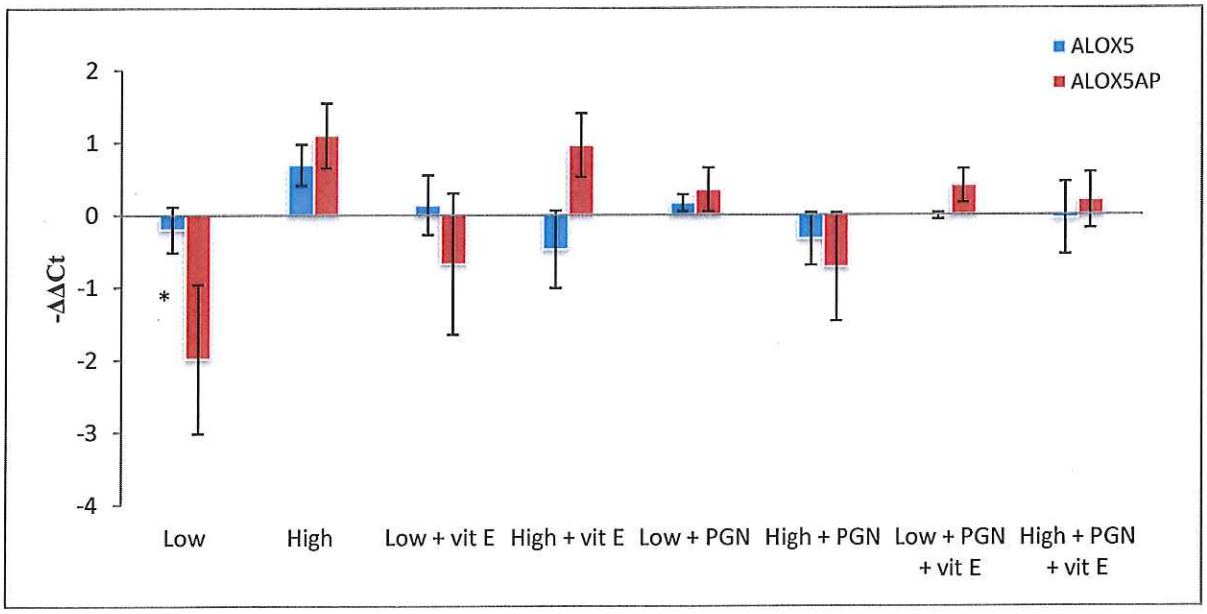

Figure 4.22 : Comparision between eicosanoid markers, analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Significance difference from control is indicated with a star (*). Control= all pooled treatment groups.

ALOX5 and ALOX5AP are genes involved in the eicosanoid metabolism. We analysed ALOX5-1 where ALOX5 reveals two isoforms were shown to respond differently to LPS in preadipocytes (Skugor et al., 2010).

Of note is that ALOX5AP showed more prominent response that ALOX5 and that is was higher on average in the groups under high omega-3 FAs treatment with highest expression in the group High. Response to PGN differed under low omega-3 FAs treatment (up-regulated) and high omega-3 FAs treatment (down-regulated) compared to the equalised control.

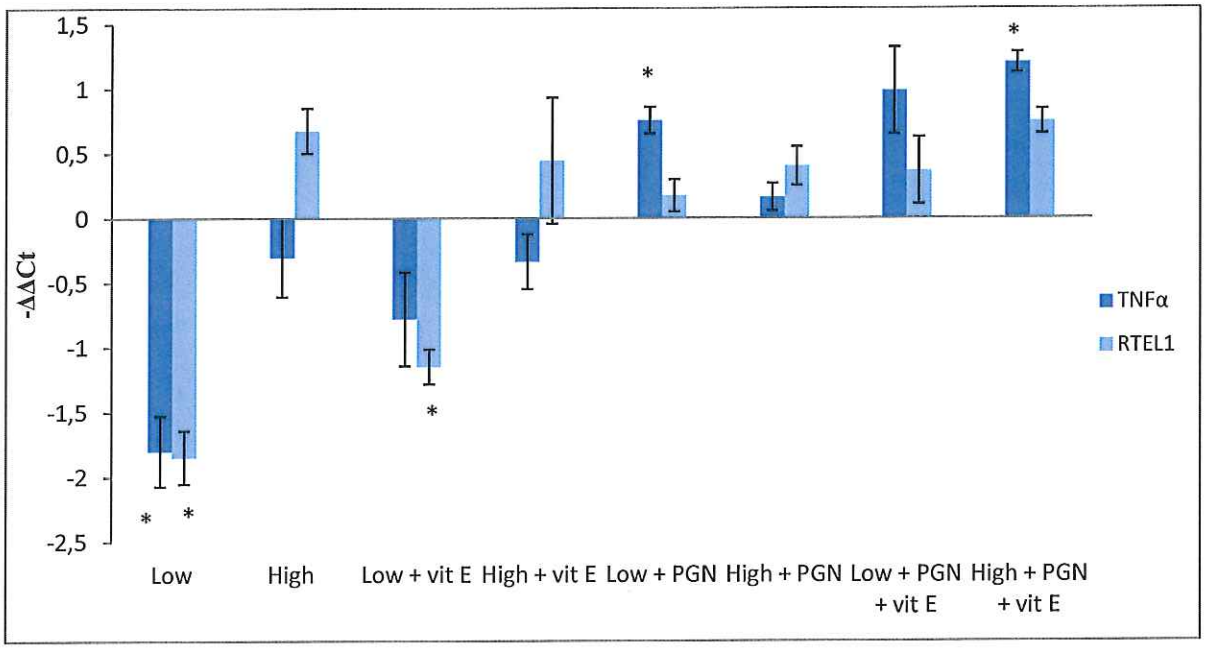

Figure 4.23: Comparision between pro-inflammatory cytokine and its receptor analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Significance difference from control is indicated with a star (*). Control= all pooled treatment groups.

TNFα is a pro-inflammatory cytokine and RTEL1 is a TNFα decoy receptor; thus, it reduces the effects of TNFα. The expression of both genes was lowest under the low omega-3 FAs and low omega-3 FAs and vitamin E treatments compared to all other groups. Higher omega-3 FAs dose positively affects the expression of TNFα but on average, an anti-inflammatory status seems to be maintained by an even higher induction of RTEL1.

Of note is the effect of omega-3 FAs dose on the TNFα and RTEL1 pair under PGN stimulation: high omega-3 FAs treatment appears to have the anti-inflammatory effect as the TNFa/RTEL1 ratio decreases. An important observation: vitamin E does not change the ratio of the two opposing genes but equally enhances the expression of both; this is a good illustration of how important it is to include more genes that are involved in the regulation of the same pathway; if TNFα was the only biomarker used to assess inflammation, it would appear that vitamin E increases the pro-inflammatory status in cells.

4.7. Combined/average effects of pro-and anti-inflammatory effects

When adipose cells are exposed with omega-3 FAs, vitamin E and immunostimulants, conjugated effects are supposed to happen. However, inductions of many pro-inflammatory responses are counted by anti-inflammatory mediator simultaneously. Therefore, to consider genes in combinations is one of the helpful approaches.

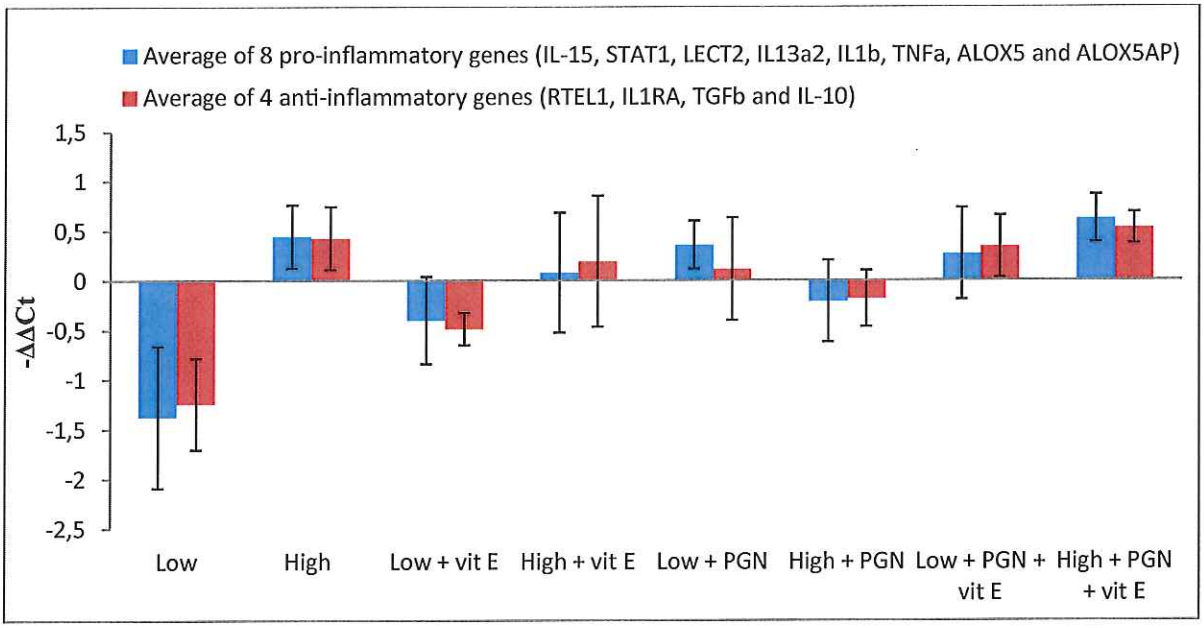

Figure 4.24: Comparision between pro and anti-inflammatory cytokines analysed with qPCR in maturing adipocytes under eight treatment groups. Data are presented as -ΔΔCt ±SE (n=5). The significance level has been set at $p \leq 0.05$. Control= all pooled treatment groups.

Under low omega-3 FAs treatment, analysed pro-inflammatory genes show lowest expression; they are, as expected, induced by the PGN treatment.

Interestingly, a comparable level of induction is observed in the High group. This could be related to newly assigned roles in energy-metabolism that many (if not all) immune-related genes are shown to possess. Omega-3 FAs negatively regulate lipid deposition and increase burning of fat (catabolism); most likely, in fish, as in mammals, genes traditionally considered to have functions exclusively in immune processes might also be involved in energy metabolism. Our previous work also points in this direction.

However, under high omega-3 fatty acids' dose, vitamin E appears to increase the level of expression of pro-and anti-inflammatory genes in comparison to low. This could be due to increased loading of adipocytes with fat and in this case, enhancement of processes involved

in lipid deposition probably needs to be balanced out by the opposite action of products encoded by immune-related genes.

However, an ability of vitamin E to affect the change in expression in opposite directions under low and high omega-3 FAs settings implies a "balancing" role for this vitamin at times when energy levels fluctuate.

As discussed in the text above (Fig 4.7) high expression level of pro-inflammatory genes observed in the High+PGN+vitamin E group is counteracted by the equally up-regulated expression of anti-inflammatory genes.

Pro-inflammatory effect of PGN appears to have Low+PGN which in turns decreased by expression of anti-inflammatory genes. Both pro- and anti-inflammatory genes are comparatively shown to have higher expression in combination with PGN and vitamin E under high omega-3 FAs treatment. However, anti-inflammatory effects of vitamin E appear in high +vitamin E where Low+vitamin E influences down-regulation. However, high and low omega-3 FAs and in combination of PGN affects the expression of pro- and anti-inflammatory genes by opposite directions.

CHAPTER V

DISCUSSION

Very limited research has been performed on how different doses of omega-3 FAs effect fish adipose biology. It is known however that adipocytes play a key role in immune system and energy metabolism. Omega-3 FAs, especially EPA and DHA, prevent the development of obesity in mammals by reducing the growth of visceral adipose tissue (Ruzickova et al., 2004; Parrish et al., 1990). The exact mechanism behind the adipose tissue lowering effect of omega-3 FAs is not yet known, but there are likely to be several processes involved in this phenomenon. One mechanism may be that the omega-3 FAs inhibit the late phase of adipocyte differentiation through the suppression of some genes, such as PPARγ and C/EBPα that regulate terminal differentiation (Madsen et al., 2005). Other proposed mechanisms are that HUFAs suppress FA synthesis and/or induce mitochondrial FA β-oxidation, leading to the utilization of stored lipids and a reduction in adipocyte size (Flachs et al., 2005; Baillie et al., 1999). However, it has recently been suggested that the loss of adipose tissue may also be caused by a decrease in adipocyte number, and that this occurs by, for example, apoptosis (Kim et al., 2006) induced by oxidative stress. In this thesis I have tested the hypothesis that EPA and DHA exert an important role in the biology of differentiating adipocytes in Atlantic salmon. Furthemore, the effects of vitamin E and PGN, alone and in combination with different doses of HUFAs were studied in relation to how they affect the process of adipogenesis. Cultured adipocytes were exposed to different doses of omega-3 FAs and their combinations with either vitamin E, PGN or vitamin E+PGN together.

5.1. Effects of different doses of omega-3 FAs, vitamin E and immunostimulant (peptidoglycan) on Adipogenesis

C/EBPα expression was arrested with high doses of omega-3 FAs while low doses up-regulated C/EBPα. Cells treated with vitamin E are characterized by the highest deposition of TAG and a marked expression of the master adipogenic regulator C/EBPα (Todorcevic et al., 2010a). Up-regulation of C/EBPα revealed that vitamin E acts as a potent pro-adipogenic vitamin under the low omega-3 FAs treatment. Immunostimulant PGN, which was used in this study decreased expression of C/EBPα and genes involved in lipid metabolism (FAS), in agreement with previous findings where LPS was used as immunostimulant (Skugor et al., 2010).

5.2. Effects of different doses of omega-3 FAs, vitamin E and immunostimulant (peptidoglycan) on lipogenesis and energy storage

Lipid peroxidation can be minimized by vitamin E; PUFAs are known for their suppressive effect on lipogenic enzyme FAS (Hyekyeong et al., 2002). In rodent hepatocytes, the expression of FAS is downregulated by the generation of PUFA peroxidation products (Foretz et al., 1999) and eicosanoid metabolites (Mater et al., 1999). However, FAS is significantly up-regulated in liver of mice injected with LPS (Chen et al., 2011). In our study, when cells were incubated with vitamin E and PGN together, high dose of omega-3 FAs mediated an increase in FAS expression while low doses exerted a suppressive effect.

HSD17B4 and plasma proteins regulate the availability of steroids by controlling the amount of active steroids accessible to receptors and available for metabolism (Maren et al., 2008). The highest levels of HSD17B4 mRNA transcription and specific activity are found in liver and kidney followed by ovary and testes in human (Launoit and Adamski, 1999). Our results suggest that HSD17B4 expression increases with higher doses of omega-3 FAs, and with vitamin E. The effects of all treatments on HSD17B4were generally weak (in the range of -0.2 to 0.2 log ER).

Tissue distribution and physiological studies of PGC-1α suggest that its principal *in vivo* roles are to promote cold-induced thermogenesis, mitochondrial biogenesis, hepatic gluconeogenesis, and fatty acid beta-oxidation. PGC-1α is expressed in the white adipose tissue of both humans and rodents. In rodents, it has been suggested that PGC-1α partly mediates leptin-induced conversion of white adipocytes from fat storing to fat oxidizing cells (Semple et al., 2004). Increases in PGC-1α levels, can be induced by physiological stimuli, alter the intramuscular lipids and improve fatty acid oxidation, insulin signaling and insulin-stimulated glucose transport, albeit to different extents in lean and insulin resistant muscle (Benton et al., 2010). It is very interesting that PGC-1α overexpression increases fatty acid and fatty acid incorporation into triacylglycerol in primary human skeletal muscle cells (Leslie et al., 2010). Our results suggest that high omega-3 FAs doses decrease fatty acid oxidation. Down-regulation of gene was also observed in combination of high omega-3 FAs with the PGN treatment.

UGT prevents the accumulation of potentially toxic compounds and/or their subsequent bioactivation to more toxic intermediates. Higher activity of UGT is likely to be found in

microsomes from rat fed with high n-3 fatty acids (Janko et al., 2003). Our result suggested that low level of omega-3 FAs doses may be more involved in detoxification of eicosanoids by expressing UGT from Atlantic salmon adipose tissues. Vitamin E altered the expression the UGT level of expression where high doses of omega-3 FAs treatments and while incorporation of vitamin was almost same effects. Significant decreases in hepatic UGT activities are reported after LPS administration in rats (Chen et al., 1992) and our result was in agreement with it.

5.3. Defense mechanisms of adipose tissue to intracellular oxidation

In order to understand more about how oxidative process are involved in the regulation of adipose tissue in fish, we followed up studies of how GPx-2 gene code for as intracellular antioxidant. It is known that high doses of EPA and DHA have been documented in oxidative stress (OS) in Atlantic salmon (Todorcevic et al., 2008a). Our results from the *in vitro* trials of adipocytes are compatible with this, and showed that EPA and DHA induces GPx-2 in salmon adipose tissue and may thus be one factor influencing lipid storage in salmon adipose tissue. For deposition of TAG, inhibition of ROS is required. Our result (fig. 4.6) revealed high doses of EPA+DHA with vitamin E induces significant level of GPx-2 which may help to protect adipose cell from ROS. However, moderate levels of EPA and DHA in fish diets reduce lipid accumulation by increasing the FA β-oxidation capacity in adipose tissue, the very high dietary EPA and DHA levels damage mitochondrial membranes so that they fail to function due to oxidative damage, leading to the induction of apoptosis (Todorcevic et al., 2008b). Moreover, it may say from figure 4.6, vitamin E shows antioxidant properties with high doses of omega-3 FAs treatments (Burton, 1998) and non-antioxidant properties with low (down-regualtion of GPx-2) omega-3 FAs treatments (Boscoboinik et al., 1991). However, there is limited knowledge about the proper balance between antioxidants and other ingredients in fish feed. In addition, it was found that increased levels of dietary HUFAs require increasing dietary supplementation with antioxidants in order to prevent oxidative damage (Stephan et al., 1995).

5.4. Effect of omega-3 FAs, vitamin E and immunostimulant (peptidoglycan) to pro inflammatory responses

TNF-α mRNA is identified and characterized in rainbow trout (*Oncorhynchusmykiss*) (Laing et al., 2001), brook trout (*Salvelinusfontinalis*) (Bobe& Goetz 2001), carp (*Cyprinuscarpio*) (Saeji et al.,. 2003) and sea bream (*Sparusaurata*) (Garcia- Castillo et al., 2002).

The effects of TNF-α on lipid metabolism include stimulation of lipolysis in human adipocytes (Zhang et al., 2002), inhibition of the expression of enzymes involve in lipogenesis such as acetyl-CoA carboxylase and fatty acid synthase, and inhibition of lipoprotein lipase (LPL) activity (Semb et al., 1987, Grunfeld et al., 1989).
Furthermore, TNF-like activity has been found in supernatants of rainbow trout macrophages stimulated with LPS. These supernatants are able to enhance neutrophil migration and macrophage respiratory burst activity (Qin et al., 2001).

TNF-α action on adipocytes can directly alter lipid metabolism through inhibition of FFA uptake and lipogenesis and stimulation of FFA release via lipolysis (William and Jaswinder, 2007). In this way, adipose tissue-derived TNF-α can contribute to the development of dyslipidaemia and resultant metabolic complication (William and Jaswinder, 2007). Moreover, TNF-α can regulate the production of other pro-inflammatory cytokines (eg. IL-6 and IL-1) and thereby further and/or amplify its effects on peripheral organs. Skugor et al., (2010) showed, TNF-α and a suite of TNF-dependent genes are up-regulated with immunostimulant (LPS) and our result also agreed with PGN as immunostimulant in Atlantic salmon. Omega-3 FAs decrease TNF-α secretion by RAW 264.7 macrophages *in vitro* and the effects of omega-3 FAs on lipopolysaccharide induced TNF-α by murine primary resident and elicited peritoneal macrophages and by RAW 264.7 macrophages which supports our findings. Furthermore, lipoprotein lipase (LPL) activity in adipose tissue of LPS injected fish is lower which suggests TNF- α plays an important role in the control of lipid metabolism in rainbow trout by stimulating lipolysis *in vitro* and *in vivo* and by down-regulating LPL activity of adipose tissue *in vivo* (Albalat et al., 2005).In addition, dietary fish oil increases TNF-α secretion by murine splenocytes after stimulation with LPS (Albers et al., 2002; Barber et al., 2005).

STAT1 mediates gene expression in response to cytokines and growth factors. Activation of STAT1 is achieved through its tyrosine phosphorylation, a process that involves Jak tyrosine

kinases (Mowen and David, 2000). However, nitration of unsaturated fatty acid suppresses the activation of proinflammatory STAT signaling induced by LPS in RAW264.7 macrophages (Tomonaga et al., 2008). But, our finding showed the trend of induction of STAT1 by immunostimulant, PGN in combination with omega-3 FAs in Atlantic salmon maturing adipocytes. STAT1 was significantly decreased with low doses of omega-3 FAs which could be effect of omega-3 FAs on tyrosine phosphorylation.

LECT2 is a potent neutrophil chemoattractant, which has effect on development of chondrocytes and osteoblasts (Hiraki et al., 1996; Mori et al., 1997). LECT2 mRNA is predominately expressed in liver and spleen, showing lower expression in kidney, intestine, heart and brain in Trout (Kokkinos et al., 2005). LECT2 was significantly induced with high doses of omega-3 FAs. LECT-2, is extremely high sensitivity to bacterial infections in fish (Lin et al., 2007). High omega-3 FAs may influence to induce pro-inflammatory gene like LECT2. In case of low omega-3 FAs doses, immunostimulant helped to induce LECT2 which shows pro-inflammatory response.

IL-15 is a T-cell growth factor and revealed a decrease of intestinal IL-15 expression in rat intestinal intraepithelial lymphocytes (IELs) with fish oil treatments, enriched with EPA and DHA (Wang et al., 2007). On the other hand, our result was agreed with low doses of omega-3 FAs treatments in Atlantic salmon maturing adipocytes. In agreement with Ulaganathan et al., (2009), IL-15 level was decreased with vitamin E treatments.

Omega-3 FAs have strong anti-inflammatory effects; suppress IL-1β, TNFα and IL-6 (Artemis, 2008). Our result was resembled with those findings except IL-1β was upregulated with with high doses of omega-3 FAs.

Decoy receptor IL-13Rα2 was down-regulated by vitamin E treatment in mature adipocytes from Atlantic salmon (Todorcevic et al., 2010a). But our results revealed vitamin E more non-antioxidant actions with high doses of omega-3 FAs than low omega-3 FAs (Fig. 4.8). High doses of omega-3 FAs showed anti-inflammatory actions could include signaling via IL4 and IL13 cascade, which was likely promoted by the down-regulation of the decoy receptor IL-13Rα. However, low doses of omega-3 FAs treatments revealed insignificant inflammatory response. T-cell stimulant phytohaemagglutinin increased the expression of IL-4Rα1 and IL-4Rα2 in rainbow trout (Wang et al., 2011).

But PGN as immune stimulant showed down-regulation of IL-13Rα2 in Atlantic salmon maturing adipocytes with high and low doses of omega-3 FAs treatments. Upregulation of ALOX5 and/or FLAP activity enhances production of leukotriene and membrane lipoperoxides (Maccarrone et al., 2001; Dixon et al., 1990). In the presence of 5-lipoxygenase-activating protein, 5-lipoxygenase converts arachidonic acid to leukotrienes, which is inflammatory mediators in diseases such as asthma, cardiovascular diseases, and many other chronic inflammatory conditions (Rubin and Mollison, 2007). mRNA levels for the ALOX5 are significantly increased in human atherosclerotic plaque (Mehrabian et al., 2008) where very few has been known on fish. Omega-3 FAs may interfere with the arachidonic acid cascade by inhibiting ALOX5 (Taccone-Gallucci et al., 2006). Expression and activity of ALOX5 is downregulated in tumor xenograft fed curcumin combined with fish oil diet when compared to individual diets (Swamy et al., 2008). Low doses of omega-3 FAs treatment were in agreement with authors. Up-regulation of ALOX5 may also be related to the increased mitochondrial damage and apoptosis of peripheral blood mononuclear cells (PBMCs) observed in end-stage renal disease (ESRD) patients (Taccone-Gallucci et al., 2006). PUFAs are transformed into leukotrines by ALOX5 from Atlantic salmon preadipocytes and immune stimulant (LPS) induced expression of genes, involved in eicosanoid metabolism (Skugor et al., 2010). ALOX5 activity and related oxidative stress are significantly (although not completely) suppressed by vitamin E in peripheral blood mononuclear cells (PBMCs) of hemodialysis patients (Maccarrone et al., 2003), in potato tubers (Pallu et al., 2001), in rat polymorphonuclear leukocytes (Chan et al., 1989), in human monocytes (Devaraj and Jialal, 1999). But our result only was supported with high+vitamin E treatments.

5.5. Effect of omega-3 FAs, vitamin E and immunostimulant (peptidoglycan) to anti-inflammatory responses

RTEL1, decoy receptor antagonist of TNF-α is commonly co-regulated with TNF-α to prevent damage from excessive activity of this cytokine. RTEL1 expression is increased 5.4 folds in Atlantic salmon preadipocytes (Skugor et al., 2010) compare with studies found 1000-fold induction or greater. RTEL is likely to act as an important immune-regulating factor in inhibiting the apoptosis-inducing effect of TNF in the skin of conger eel (Shigeyuki et al., 2008).

The elevated level of IL-1RAis found with PUFAs treatments in neonatal cardiomyocytes and hearts of adult rats (Kaplinskyi et al., 2009). Our result was supported with high omega-3 FAs treatments. IL-1RAexpression was found to be increased by low+vitamin E treatments where IL-1β is found to be decreased by vitamin E (Azzi et al., 2004). However, Lin et al., 2002 also shows vitamin E as immune modulatory by increasing gene expressions of IL-1 receptor antagonist. Immune stimulant (LPS) is found to be increased level of IL-1RA (Cartmell et al., 2001) which is supported with Low+PGN treatments.

Increased TGF-β and decreased oncogene expression by omega-3 FAs in the spleen delays onset of autoimmune disease in mice. TGF-β is found to be increased by omega-3 FAs in rat (Fermandes et al., 1994), hey cell lines (Sharma et al., 2005), epithelial ovarian cancer (Sharma et al., 2005). Our result was found to be agreed with high doses of omega-3 FAs treatments.

Vitamin E dietary supplementation inhibits TGF-β1 gene expression in the rat liver (Parola et al., 1992; Kuemmerle et al., 1997). However, treatment with vitamin C or E prevented the increase in glomerular TGF-β immunoreactivity (Craven et al., 1997) and diabetes-induced over expression of TGF-β1 mRNA (Cojocel et al., 2005). Vitamin E inhibits TGF-β release in mesangial cells (José-Antonio et al., 2004). We found opposite effects of vitamin E with omega-3 FAs treatments. Activation of monocytes with immunostimulant (LPS) stimulates the secretion of TGF-β; however, the production of TGF-β by neutrophils is not altered by treatment with immune stimulant (LPS) (Gary et al., 2005). Low doses of omega-3 FAs with PGN was found to be showed stimulation of TGF-β.

In agreement with our result omega-3 FAs have strong anti-inflammatory effects, suppress IL-6 (Artemis, 2001; Barbosa et al., 2010). Vitamin E administration attenuates IL-6 responses in skeletal and cardiac muscle to an inflammatory challenge induced by systemic LPS (Huey et al., 2008).

Omega-3 FAs does not affect IL-10 secretion by the cells but decrease the number of cells secreting IL-10 *ex vivo*, possibly by affecting cell recruitment, maturation or proliferation by RAW 264.7 macrophages (Skuladottir et al., 2007).An increase in IL-10 levels is observed in mice fed the omega-3 FAs diet and suggests that omega-3 FAs down-regulate the inflammatory response by enhancing IL-10 expression (Sierra et al., 2004).

In vitro *Lactobacillus salivarius* PGN induces IL-10-producing dendritic cells (Fernandez et al., 2011) and indicates the anti-inflammatory actions when induced by LPS (Deirdre et al., 2006). Skeletal and cardiac IL-6 mRNA and protein is significantly elevated by LPS, but responses are significantly lower in vitamin E compared with placebo-treated mice (Huey et al., 2008). Our result also showed PGN was likely to help elevate IL-6 expression and vitamin E effects with low doses of omega-3 FAs.

5.6. Gene expression effects of omega-3 FAs, vitamin E and immunostimulant (peptidoglycan)

Omega-3 FAs have anti-inflammatory effects, suppress IL-1β, TNF-α and IL-6 (Simopoulos, 2006). The fish oil diet lowers the level of cytokines (IL-6, IL-10 and TNF-α) and lipid mediators in mice. Adding 500 IU of vitamin E to the fish oil diet further lowers the levels of IL-6, IL-10 and TNF-α which suggest the beneficial effects of fish oil can be enhanced by the addition of 500 IU of vitamin E in the diet (Venkatraman and Chu, 1999). Our result was in agreement with authors except IL-6 with Low+vitamin E treatments. However, IL-10 inhibits TNF-α production and neutrophil activity in LPS induced acute lung injury in mouse a model (Gen, 2000). IL-10 probably helped to reduce the level of TNF-α with low+ a PGN treatment which is in line with Gen, 2000. Vitamin E has immunomodulatory effects by lowering the levels of IL-10 in mice (Subir et al., 2009). The significantly increases of TNF-α and IL-10 concentration suggest that vitamin E supplementation represents an important factor in the defences against oxidative stress and muscle damage by eccentric exercise (EE) in humans (Luciano et al., 2009) and our results also in agreement with maturing adipocytes from Atlantic salmon. Dietary fish oil increases the number of splenocytes secreting TNF-α and IL-10, following stimulation with immunostimulant (LPS) (Dagbjort and Ingibjorg, 2007) which is agreed with our result except High+PGN treatments. PGN are potent inducers of IL-1beta and IL-6 gene expression (MacKenzie et al., 2010). However, PGN- treated in human pulp-derived fibroblasts and permanent teeth induces IL-1β and IL-1β mRNA levels (Yasuhiro et al., 2002). Nonetheless, PGN stimulated C/EBPδ protein and mRNA expression in mouse macrophages RAW 264.7 cells (Huang et al., 2007). Moreover, vitamin E has multidimensional functional profile to regulate uptake and degradation of tocopherols, lipid uptake and atherosclerosis, modulate extracellular proteins and inflammation and implicate in cell signalling & regulation (Azzi et al., 2004).

CHAPTER VI

CONCLUSION

Different doses of omega-3 FAs, vitamin E and PGN affected a great number of studied genes with roles in adipogenesis, energy metabolism, oxidative stress and immunity.

Atlantic salmon possesses numerous mechanisms to protect itself from adverse effects of excessive lipid storage. Different doses of omega-3 FAs showed pivotal role in lipid accumulation and intracellular antioxidant capacity.

Vitamin E appears to act as an antioxidant. Vitamin E has important role in controlling immunity and adipogenesis.

PGN, as expected, induced expression of several immune genes. Interestingly, PGN and high doses of omega-3 FAs had almost same level of immune genes induction.

Inflammation is a key factor underlying inflammatory mediator's responses. Nonetheless, high expression level of pro-inflammatory genes was counteracted by the equal up-regulated expression of anti-inflammatory genes. Immunostimulant was involved to express almost all respective pro-inflammatory genes with low omega-3 FAs doses.

Combinations of treatments produced some unexpected results. This suggests to conduct further research is necessary to study them together in the future as their single effects can be very different from the situation when they act together.

REFERENCE LIST

Aarnes M, Schonberg S, and Grill V. 2002. Fatty acids potentiate interleukin-1beta toxicity in the beta-cell line INS-1E. *Biochem Biophys Res Commun.* **296:**189-93.

Adachi K, Endo H, Watanabe T, Nishioka T, and Hirata T. 2005. Hemocyanin in the exoskeleton of crustaceans: enzymatic properties and immunolocalization. *Pigment Cell Res.* **18:** 136-143.

Ajuwon KM, Kuske JL, Anderson DB, Hancock DL, Houseknecht KL, Adeola O, and Spurlock ME. 2003. Chronic leptin administration increases serum NEFA in the pig and differentially regulates PPAR expression in adipose tissue. *J. Nutr. Biochem.* **14:** 576–583.

Albalat A, Liarte C, MacKenzie S, Tort L, Planas JV, and Navarro I. 2005. Control of adipose tissue lipid metabolism by tumor necrosis factor- alpha in rainbow trout (*Oncorhynchus mykiss*). *J. Endocrinol.* **184:** 527-534.

Albers R, Bol M, Bleumink R, Willems A, Blonk C, and Pieters R. 2002. Effects of dietary lipids on immune function in a murine sensitisation model. *Br. J. Nutr.* **88:** 291–299.

Alvarez B, Alvarez MA, Amor I, García ME, and Ruiz MA. 2011. A Thiophosphinidene Complex as a Vehicle in Phosphinidene Transmetalation: Easy Formation and Cleavage of a P-S Bond. *Inorg Chem.* **50:** 10561-10563.

Amlashi AS, Falahatkar B, Sattari M, Tolouei, and Gilani MH. 2011. Effect of dietary vitamin E on growth, muscle composition, hematological and immunological parameters of sub-yearling beluga Huso huso L. *Fish & Shellfish Immunology.* **30:** 807-814.

Amri E, Ailhaud G, and Grimaldi PA. 1994. Fatty acids as signaling molecules: Involvement in the differentiation of preadipose to adipose cells. *J. Lipid Res.* **35:** 930–937.

Antonicelli F, Brown D, Parmentier M, Drost ME, Nik Hirani, Irfan R, Ken D, and William M. 2004. Regulation of LPS-mediated inflammation in vivo and in vitro by the thiol antioxidant Nacystelyn. *Am J Physiol Lung Cell Mol Physiol.* **286:**1319-1327.

Arnesen P, Krogdahl Å, and Kristiansen IØ. 1993. Lipogenic enzyme activities in liver of Atlantic salmon (*Salmo salar*, L.). *Comp. Biochem. Physiol. B.* **105:** 541–546.

Artemis PS. 2001. Omega-3 Fatty Acids in Inflammation and Autoimmune Diseases. *J. Am. Coll Nutr.* **21:** 495-505.

Artemis PS. 2008. The omega-6/omega-3 fatty acid ratio, genetic variation, and cardiovascular disease. *Asia Pac J Clin Nutr.* **17:** 131-134.

Azzi A, and Stocker A. 2000. Vitamin E: non-antioxidant roles. *Progress in Lipid Research.* **39 (3):** 231-255.

Azzi A, Gysin R, Kempna P, Munteanu A, Negis Y, Villacorta L, Visarius T, and Zingg J. 2004. Vitamin E Mediates Cell Signaling and Regulation of Gene Expression. Ann. N.Y. Acad. Sci. **1031:** 86–95.

Baille RA, Takada R, Nakamura M, and Clarke SD. 1999. Coordinate induction of peroxisomal acyl-CoA oxidase and UCP-3 by dietary fish oil: a mechanism for decreased body fat deposition. *Prostaglandins Leukotrines and Essential Fatty Acids.* **60:** 351-356.

Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A, and Evans RM. 1999. PPARγ is required for placental, cardiac, and adipose tissue development. *Mol Cell.* **4:** 585–595.

Barber MD, Fearon KC, and Ross JA. 2005. Eicosapentaenoic acid modulates the immune response but has no effect on a mimic of antigen-specific responses. *Nutrition.* **21:** 588–593.

Barbosa VM, Elizabeth AM, Conceição C, Estevão L, and Philip CC. 2010. Effects of a fish oil containing lipid emulsion on plasma phospholipid fatty acids, inflammatory markers, and clinical outcomes in septic patients: a randomized, controlled clinical trial. *Biomed Central.***14**:1-11.

Bartov I, and Bornstein S. Stability of abdominal fat and meat of broiler: combined effect of dietary vitamin E and synthetic antioxidants. *Poultry Sci.* **60:** 1840–1845.

Belo MA, Schalch SH, Moraes FR, Soares VE, Otoboni AM, and Moraes JE. 2005. Effect of dietary supplementation with vitamin E and stocking density on macrophage recruitment and giant cell formation in the teleost fish, Piaractus mesopotamicus. *J Comp Pathol.***133(2-3):**146-54.

Bendich A, Diapolito P, Gabriel E, and Machun LJ. 1984. Interaction of dietary vitamin C and vitamin E in guinea pig immune responses to mitogens. *J. Nutr.***114:** 1588-1593.

Benton CR, Holloway GP, Han XX, Yoshida Y, Snook LA, Lally J, Glatz JFC, Luiken JJFP, Chabowski A, and Bonen A. 2010. Increased levels of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) improve lipid utilisation, insulin signalling and glucose transport in skeletal muscle of lean and insulin-resistant obese Zucker rats. *Diabetologia.* **10:** 1007.

Berczi I, Bertok L, and Bereznai T. 1966. Comparative studies on the toxicity of Escherichia coli lipopolysaccharide endotoxin in various animal species. *Can. J. Microbiol.* **12:** 1070–1071.

Bernardo LW. 2000. Subcutaneous and Visceral Adipose Tissue: Their Relation to the Metabolic Syndrome. *Endocrine Reviews* . **21(6):** 697–738.

Bobe J , and Goetz FW. 2001. Molecular cloning and expression of a TNF receptor and two ligands in the fish ovary. *Comparative Biochemistry and Physiology B. Biochemistry and Molecular Biology.* **129:** 475–481.

Boggio SM, Hardy RW, Babbitt JK, and Brannon EL. 1985. The influence of dietary lipid source and alpha-tocopherol acetate level on product quality of rainbow trout (*Salmo gairdneri*). *Aquaculture.* **51:** 13–24.

Boscoboinik D, Szewczky A, Henset C, and Azzi A. 1991. Inhibition of cell proliferation by alpha-tocopherol. *J. Biol. Chem.* **266:** 6188–6194.

Breitling R, Marijanovic Z, Perovic D, and Adamski J. 2001. Evolution of 17β-HSD type 4, a multifunctional protein of β-oxidation. *Molecular and Cellular Endocrinology.* **171:** 205-210.

Burton B. 1998. Inborn errors of metabolism in infancy: a guide to diagnosis. *Pediatrics.* **102 (6):** E69.

Burton GW, and Ingold KU. 1981. Autoxidation of biological molecules: The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants in vitro. *J. Am. Chem. Soc.* **103:** 6472–6477.

Burton GW. 1998. Human plasma and tissue alpha-tocopherol concentrations in response to supplementation with deuterated natural and synthetic vitamin E. *Am. J. Clin Nutr.* **67:** 669-684.

Cancello R, and Clement K. 2006. Is Obesity an inflammatory illness? Role of low-grade inflammation and macrophage infiltration in human white adipose tissue. *Bjog-An International Journal of obstetrics and gynaecology.* **113:** 1141-1147.

Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, Coupaye M, Pelloux V, Hugol D, and Bouillot JL. 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:** 2277–2286.

Carriere A, Carmona MC, Fernandez Y, Rigoulet M, Wenger RH, Penicaud L, and Casteilla L. 2004. Mitochondrial reactive oxygen species control the transcription factor CHOP-10/GADD153 and adipocyte differentiation: A mechanism for hypoxia-dependent effect. *J. Biol. Chem.* **279:** 40462–40469.

Cartmell T, Luheshi GN, Hopkins SJ, Rothwell NJ, and Poole S. 2001. Role of endogenous interleukin-1 receptor antagonist in regulating fever induced by localised inflammation in the rat. *The Journal of Physiology.* **531:** 171-180.

Cha SH, Fukushima A, Sakuma K, and Kagawa Y. 2001. Chronic docosahexaenoic acid intake enhances expression of the gene for uncoupling protein 3 and affects pleiotropic mRNA levels in skeletal muscle of aged C57BL/6NJcl mice. *J. Nutr.* **131:** 2636–2642.

Chan AC, Tran K, Pyke DD, and Powell WS. 1989. Effects of dietary vitamin E on the biosynthesis of 5-lipoxygenase products by rat polymorphonuclear leukocytes (PMNL). *Biochim Biophys Acta.* **17:** 265-269.

Chatelain E, Boscoboinik DO, Bartoli GM, Kagan VE, Gey FK, Packer L, and Azzi A. 1993. Inhibition of smooth muscle cell proliferation and protein kinase C activity by tocopherols and tocotrienols. *Biochim. Biophys. Acta.* **1176:** 83-89.

Chen Xi, Zhang C, Zhao M, Shi Chang-E, Ren-Min Z, Wang H, Zhao H, Wei W, Jia-BL, and Xiang XD. 2011. Melatonin alleviates lipopolysaccharide-induced hepatic SREBP-1c activation and lipid accumulation in mice. *J. Pineal Res.* **51:** 416–425.

Chen YL, Florentin I, Batt AM, Ferrari L, Giroud JP, and Chauvelot-Moachon L. 1992. Effects of interleukin-6 on cytochrome P450-dependent mixed-function oxidases in the rat. *Biochem Pharmacol.* **44:**137–148.

Cho SH, and Choi YS. 1994. Lipid peroxidation and antioxidant status is affect by different vitamin E levels when feeding fish oil. *Lipids.* **29:** 47–52.

Choy L, and Derynck R. 1998. The type II TGF-β-receptor-associated protein TRIP-1 acts as a modulator of the TGF-β response. *J. Biol. Chem.* **273:** 31455-31462.

Clement K, Viguerie N, Poitou C, Caretter C, Pelloux V, Curat C, Sicard A, Rome S, Benett B, and Benis A. 2004. Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. *FASEB J.* **18:** 1657–1669.

Cojocel C, Al-Maghrebi M, Thomson M.S, Rawoot P, and Raghupathy R. 2005. Modulation of the transforming growth factor beta1 by vitamin E in early nephropathy, *Med Princ Pact.* **14:** 422-429.

Combs GF, and Regenstein JM. 1980. Influence of selenium, vitamin E and ethoxyquin on lipid peroxidation in muscle tissues from fowl during low temperature storage. *Poultry Sci.* **59:** 347–351.

Couet C, Delarue, Ritz P, Antoine JM, and Lamisse F. 1997. Effect of dietary fish oil on body fat mass and basal fat oxidation in healthy adults. *Int J Obes.* **21**: 637-643.

Cowey CB, and Walton MJ. 1989. Intermediary metabolism. In: Halver, J.E. (Ed.), *Fish Nutrition, 2nd edn.* 259–329.

Cowey CB. 1993. Some effects of nutrition on flesh quality of cultured fish. *Fish Nutrition in Practice. Proc. IV Int.* **61:** 227–236.

Craven PA, DeRubertis FR, Kagan VE, Melhem M, and Studer RK. 1997. Effects of supplementation with Vitamin C or E on albuminuria, glomerular TGF-beta, and glomerular size in diabetes. *Journal of the American Society of Nephrology.* **8** :1405–1414.

Dagbjort HP, and Ingibjorg H. 2007. Dietary Fish Oil Increases the Number of Splenic Macrophages Secreting TNF-a and IL-10 But Decreases the Secretion of These Cytokines by Splenic T Cells from Mice. *J. Nutr.* **137:** 665–670.

Dalen KT, Schoonjans K, Ulven SM, Weedon-Fekjaer MS, Bentzen TG, Koutnikova H, Auwerx J, and Nebb HI. 2004. Adipose tissue expression of the lipid droplet-associating proteins S3-12 and perilipin is controlled by peroxisome proliferatoractivated receptor-gamma. *Diabetes.* **53:** 1243–1252.

Dalen KT, Schoonjans K, Ulven SM, Weedon-Fekjaer MS, Bentzen TG, Koutnikova H, Auwerx J, and Nebb HI. 2004. Adipose tissue expression of the lipid droplet-associating proteins S3-12 and perilipin is controlled by peroxisome proliferator-activated receptor-gamma. *Diabetes.* **53:** 1243–1252.

Deirdre H, Roisin S, Karen E, Bernard M, and Sean C. 2006. Interleukin-10 (IL-10) but not Lipopolysaccharide (LPS) produces increased motor activity and abnormal exploratory patterns while impairing spatial learning in Balb/c mice. *Physiol Behav.* **87(5):** 842-847.

Devaraj S, and Jialal I. 1999. Alpha-tocopherol decreases interleukin-1 beta release from activated human monocytes by inhibition of 5-lipoxygenase. *Arterioscler Thromb Vasc Biol.* **19(4):** 1125-1133.

Ding ST, and Mersmann HJ. 2001a. Fatty acids modulate porcine adipocyte differentiation and transcripts for transcription factors and adipoycte-characteristic proteins. *J. Nutr. Biochem.* **12:**101–108.

Ding ST, Lapillonne A, Heird WC, and Mersmann HJ. 2003. Dietary fat has minimal effects on fatty acid metabolism transcript concentrations in pigs. *J. Anim. Sci.* **81:** 423–431.

Ding ST, Shinckel AP, Weber TE, and Mersmann HJ. 2001b. Expression of porcine transcription factors and genes related to fatty acid metabolism in different tissues and genetic populations. *J. Anim. Sci.* **78:** 2127–2134.

Dixon RA, Diehl RE, Opas E, Rands E, Vickers PJ, Evans JF, Gillard JW, and Miller DK. 1990. Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature.* **343:** 282-284.

Djordjevic B. 2009. Modulation of splenic immune responses to bacterial lipopolysaccharide in rainbow trout (*Oncorhynchus mykiss*) fed lentinan, a beta-glucan from mushroom *Lentinula edodes*. *Fish Shellfish Immunol.* **26** (2): 201–209.

Duffield J. 2003. The inflammatory macrophage: a story of Jekyll and Hyde. *Clin. Sci.* **104**: 27–*28.*

Duplus E, Glorian M, and Forest C. *2000.* Fatty Acid Regulation of Gene Transcription. *J. Biol. Chem.* **275:** 30749–*30752.*

Duplus E, Glorian, and Forest C. 2001. Fatty acid regulation of gene expression in adipogenesis and adipocyte metabolism. *New Avenues of Research in Fatty Acid Oxidation and Ketone Body Metabolism.* FAOXK Press, London. 11–16.

Eder K, Baffy N, Falus A, and Fulop AK. 2009. The major inflammatory mediator interleukin-6 and obesity. *Inflamm Res.* **58:**727-36.

Etgen GJ, Oldham BA, Johnson WT, Broderick CL, Montrose CR, and Brozinick JT. 2002. A tailored therapy for the metabolic syndrome: The dual Peroxisome Proliferator-Activated Receptor-α/γ agonist LY465608 ameliorates insulin resistance and diabetic hyperglycemia while improving cardiovascular risk factors in preclinical models. *Diabetes.* **51:**1083-1087.

Fabio SL, Jose CR, Claudio AC, Eliane BR, Claudia ON, Lila MO, and Joao FM. 2011. Supplementing alpha-tocopherol (vitamin E) and vitamin D3 in high fat diet decrease IL-6 production in murine epididymal adipose tissue and 3T3-L1 adipocytes following LPS stimulation. *Lipids in health and disease.* **10:** 37.

Fain J, Bahouth S, and Madan A. 2004. TNF-alpha release by the nonfat cells of human adipose tissue. *Int. J. Obes. Relat. Metab. Disord.* **28:** 616–622.

Farrell Ap. 2002. Coronary arteriosclerosis in salmon: growing old or growing fast? *Comparative Biochemistry and Physiology A- Molecular and integrative physiology.* **132:**723-735.

Fermandes G, Bysani C, Venkatraman JT, Tomar V, and Zhao W. 1994. Increased TGF-beta and decreased oncogene expression by omega-3 fatty acids in the spleen delays onset of autoimmune disease in B/W mice. *J. Immunol.* **152(12):** 5979-5987.

Fernández DA, Lattuca ME, Boy CC, Pérez AF, Ceballos SG, Vanella FA, Morriconi ER, Malanga GF, Aureliano DR, and Rimbau S. 2009. Energy density of sub-Antarctic fishes from the Beagle Channel. *Fish Physiol Biochem.* **35:**181–188.

Fernandez EM, Valenti V, Rockel C, Hermann C, Pot B, Boneca IG, and Grangette C. 2011. Anti-inflammatory capacity of selected lactobacilli in experimental colitis is driven by NOD2-mediated recognition of a specific peptidoglycan-derived muropeptide. *Gut Online.* **60(8):**1050-1059.

Fernyhough, ME, Okine E, Hausman G, Vierck JL, and Dodson MV. 2007. PPARγ and GLUT-4 expression as developmental regulators/markers for preadipocyte differentiation into an adipocyte. *Domest. Anim. Endocrinol.* **33:** 367–378.

Ferrante AW. 2007. Obesity-induced inflammation: a metabolic dialogue in the language of inflammation. *J. Intern Med.* **262:** 408–414.

Ferre N, Camps J, Paul A, Cabre M, Calleja L, Osada J, and Joven J. 2001. Effects of high-fat, low cholesterol diets on hepatic lipid peroxidation and antioxidants in apolipoprotein E-deficient mice. *Molecular and Cell Biochemistry*, **218:**165–169.

Flachs P, Horakova O, Brauner P, Rossmeisl M, Pecina P, Franssen-van Hal N, Ruzickova J, Sponarova J, Drahota Z, Vlcek C, Keijer J, Houstek J, and Kopecky J. 2005. Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce beta-oxidation in white fat. *Diabetologia.* **48:** 2365-2375.

Foretz MF, Foufelle, and Ferre P. 1999. Polyunsaturated fatty acids inhibit fatty acid synthase and spot-14- protein gene expression in cultured rat hepatocytes by a peroxidative mechanism. *J. Biochem.* **341:** 371–376.

Forman BM, Chen J, and Evans RM. 1997. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc. Natl. Acad. Sci.* **94:** 4312–4317.

Frayn KN, Karpe F, Fielding BA, Macdonald IA, and Coppack SW. 2003. Integrative physiology of human adipose tissue. *Int J Obes Relat Metab Disord.* **27**: 875–888.

Fried S, Bunkin D, and Greenberg A. 1998. Omental and subcutaneous adipose tissue of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J. Clin. Endocrinol. Metab.* **83:** 847–850.

Frigg W, Prabucki AL, and Ruhdel EU. 1990. Effect of dietary vitamin E levels on oxidative stability of trout fillets. *Aquaculture*. **84:** 145–158.

Fynn-Aikins KHS, Liu W, and Li H. 1992. Growth, lipogenesis and liver composition of juvenile white sturgeon fed different levels of D-glucose. *Aquaculture.* **105:** 61–72.

Garcia-Castillo J, Pelegrin P, Mulero V, and Meseguer J. 2002. Molecular cloning and expression analysis of tumor necrosis factor alpha from a marine fish reveals its constitutive expression and ubiquitous nature. *Immunogenetics* **54:** 200–207.

Gatlin DM, Bai SC, and Erickson MC. 1992. Effects of dietary vitamin E and synthetic antioxidants on composition and storage quality of channel catfish, *Ictalurus punctatus*. *Aquaculture*. **106:** 323–332.

Gen I. 2000. Effect of interleukin-10 (IL-10) on experimental LPS-induced acute lung injury. *J. Infect Chemother.* **6:** 51-60.

Goldman M, Velu T. 1995. Interleukin-10 and its implications for immunopathology. *Adv Nephrol.* **24:**79-90.

Gregoire FM, Smas CM, and Sul HS. 1998. Understanding adipocyte differentiation. *Physiol. Rev.* **78:** 783–809. Thuillier P, Bailliea R, Shaa X, and Clarke SD. 1998. Cytosolic and nuclear distribution of PPAR2 in differentiating 3T3-L1 preadipocytes. *J. Lipid Res.* **39:** 2329–2338.

Gregorie FM, Smas CM, and Slu HS. 1998. Understanding adipocyte differentiation. *Physiological Reviews.* **78:** 783-809.

Grunfeld C, Gulli R, Moser AH, Gavin LA, and Feingold KR. 1989. Effect of tumor necrosis factor administration *in vivo* on lipoprotein lipase activity in various tissues of the rat. *Journal of Lipid Research.* **30:** 579–585.

Hausman DB, DiGirolamo M, Bartness TJ, Hausman GJ, and Martin RJ. 2001. The biology of white adipocyte proliferation. *Obes. Rev.* **2:**239–254.

Henricson BE, Manthey CL, Perera PY, Hamilton TA, and Vogel SN. 1993. Dissociation of lipopolysaccharide (LPS)-inducible gene expression in murine macrophages pretreated with smooth LPS versus monophosphoryl lipid A. *Infect. Immun.* **61(6):** 2325-2333.

Herpen NAV, and Schrauwen-Hinderling VB. 2008. Lipid accumulation in non-adipose tissue and lipotoxicity. *Physiology & Behavior.* **94:** 231-241.

Hiraki Y, Inoue H, Kondo J, Kamizono A, Yoshitake Y, and Shukunami C. 1996. A novel growth-promoting factor derived from fetal bovine cartilage, chondromodulin II - Purification and amino acid sequence. *J. Biol. Chem.* **271:** 22657–22662.

Hiraki Y, Inoue H, Kondo J, Kamizono A, Yoshitake Y, Shukunami C, and Suzuki F. 1996. A novel growth-promoting factor derived from fetal bovine cartilage, chondromodulin II. *Purification and amino acid sequence.* **271(37):** 22657-22662.

Hong Q, Anders G, Hanna E, Min W, Anders W, Chi-Huey W, Ulf H, Jesper S, Goran K, Bengt S, Gabrielle P, and Jesper Z. 2006. Expression of 5-lipoxygenase and leukotriene A4 hydrolase in human atherosclerotic lesions correlates with symptoms of plaque instability. *The National Academy of Sciences of the USA.* **103:** 8161–8166.

Hotamisligil GS, Shargill NS, and Spiegelman BM. 1993. Adipose expression of TNF-alpha-Direct role in obesity-linked insulin resistance. *Science.* **259:** 87-91.

Huang YC, Chang WC, Su JG, Cai JL, Chen CC, Hung JJ, and Liu YW. 2007. Peptidoglycan enhances transcriptional expression of CCAAT/enhancer-binding protein delta gene in mouse macrophages. *J Biomed Sci.* **14(3):** 407-418.

Huey KA, Fiscus G, Richwine AF, Johnson RW, and Meador BM. 2008. *In vivo* vitaminE administration attenuates interleukin-6 and interleukin-1β responses to an acute inflammatory insult in mouse skeletal and cardiac muscle. *Exp Physiol.* **93:** 1263–1272.

Hyekyeong K, Sungwon C, Hae-Jeung L, Joo-Hee L, and Haymie C. 2002. Suppression of Fatty Acid Synthase by Dietary Polyunsaturated Fatty Acids is Mediated by Fat itself, not by Peroxidative Mechanism. *Journal of Biochemistry and Molecular Biology.* **36:** 258-264.

Ide T, Kano S, Murata M, Yanagita T, and Sugano M. 1994. Dietary modications of the biliary bile acid glycine : taurine ratio and activity of hepatic bile acid-CoA : amino acid N-acyltransferase (EC 2×3×1) in the rat. *British Journal of Nutrition.* **72:** 93-100.

Iliev DB, Roach JC, Mackenzie S, Planas JV, and Goetz FW. 2005. Endotoxin recognition: in fish or not in fish? *FEBS Letters.* **579:** 6519–6528.

Jan R. 2000. Use of Immunostimulatory Substances in Fish and Shellfish Farming. *Reviews in Fisheries Science.* **4(3):** 229-288.

Javonhey-Donzel A, Guenot L, Manpoil V, Rochette L, and Rocquelin G. 1993. Rat vitamin E status and heart lipid peroxidation: effect of dietary á-linolenic acid and marine n-3 fatty acids. *Lipids.* **28:** 651–655.

Jonsson N, Jonsson B, and Hansen LP. 1997. Changes in proximate composition and estimates of energetic costs during upstream migration and spawning in Atlantic salmon, Salmo salar. *J. Anim. Ecol.* **66:** 425-436.

José-Antonio G, Xavier M, Eva V, Ana A, Antonio S, and Juan-RM. 2004. Vitamin E attenuates biochemical and morphological features associated with development of chronic pancreatitis. *Am J Physiol Gastrointest Liver Physiol* . **287:**162-169.

Juge-Aubry CE, Somm E, Giusti V, Pernin A, Chicheportiche R, Verdumo C, Rohner-Jeanrenaud F, Burger D, Dayer JM, and Meier CA. 2003. Adipose tissue is a major source of interleukin-1 receptor antagonist: upregulation in obesity and inflammation. *Diabetes.* **52:**1104–1110.

Junko K, Atsushi I, and Takaharu M. 2003. The Effect of Dietary Fatty Acids on the Expression Levels and Activities of Hepatic Drug Metabolizing Enzymes. *J.Health Sci.* **49(2):** 105-114.

Kaplinsky SP, Shysh AM, Nahibin VS, Dosenko VIe, Klimashevskyi VM, and Moĭbenko OO. 2009. Omega-3 polyunsaturated fatty acids stimulate the expression of PPAR target genes. *Fiziolohichnyi zhurnal Kiev Ukraine.* **55**: 37-43.

Kim HK, La-Fera M, Lin J, and Baile CA. 2006. Docosahexanoic acid inhibits adipocyte differentiation and induces apoptosis in 3T3-L1 preadipocytes. *Journal of Nutrition.* **136:** 2965-2969.

Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, and Lehmann JM. 1997. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc. Natl. Acad. Sci.* **94:** 4318–4332.

Kokkinos PA, Kazantzi A, Sfyroera G, and Zarkadis IK. 2005. Molecular cloning of leukocyte cell-derived chemotaxin 2 in rainbow trout. *Fish Shellfish Immunol.* **18(5):** 371-380.

Kokta TA, Dodson MV, Gertler A, and Hill RA. 2004. Intercellular signaling between adipose tissue and muscle tissue. *Domest. Anim. Endocrinol.* **27:** 303–331.

Kuemmerle NB, Brandt RB, and Chan W. 1997. Inhibition of transforming growth factor beta 1 induction by dietary vitamin E in unilateral ureteral obstruction in rats. *Biochem Molec Med.* **61:** 82–86.

Laing KJ, Wang T, Zou J, Holland J, Hong S, Bola N, Hirono I, Aoki T, and Secombes CJ 2001 Cloning and expression analysis of rainbow trout, *Oncorhynchus mykiss*, tumor necrosis factor-alpha. *European Journal of Biochemistry.* **268:** 1315–1322.

Landrier JF, Gouranton E, Claire El, Christiane M, Patrick B, Patrick B, and Marie-Jose A. Adiponectin Expression Is Induced by Vitamin E via a Peroxisome Proliferator-Activated Receptor-γ Dependent Mechanism. *Endocrinology.* **150(12):** 5318–5325.

Launoit Y de, and Adamski J. 1999. Unique multifunctional HSD17B4 gene product:17α-hydroxysteroid dehydrogenase 4 and D-3-hydroxyacylcoenzymeA dehydrogenase/hydratase involved in Zellweger syndrome. *Journal of Molecular Endocrinology.* **22:** 227–240.

Leibowitz H, Krueger D, and Maunder L. 1980. The Framingham Eye Study monograph: an ophthalmological and epidemiological study of cataract, glaucoma, diabetic retinopathy, macular degeneration, and visual acuity in a general population of 2631 adults, 1973–1975. *Surv. Ophthalmol.* **24:** 335–610.

Leslie AC, Jill AB, Timothy RK, Deborah MM, Matthew WH, Kimberly RH, Lynis DG, and Joseph AH. 2010. Peroxisome Proliferator–Activated Receptor-γCoactivator-1α Overexpression Increases Lipid Oxidation in Myocytes From Extremely Obese Individuals. *Diabetes.* **59:**1407–1415.

Li X, and Qin J. 2005. Modulation of Toll-interleukin 1 receptor mediated signaling. *J. Mol. Med.* **83:** 258-266.

Likimani TA, and Wilson RP. 1982. Effects of diet on lipogenic enzyme activities in channel catfish hepatic and adipose tissue. *J. Nutr.* **112:** 112–117.

Lin B, Chen SW, Cao Z, Lin YQ, Mo DZ. and Zhang HB. 2007. Acute phase response in zebrafish upon *Aeromonas salmonicida* and *Staphylococcus aureus* infection: striking similarities and obvious differences with mammals. *Mol Immunol.* **44:** 295–301.

Lin H, Romsos DR, Tack PI, and Leveille GA. 1977. Influence of dietary lipid on lipogenic enzyme activities in coho salmon (*Oncorhynchus kisutch* (Walbaum)). *J. Nutr.* **107:** 846–854.

Lin KW, Chen SC, Chang FH, Kung JT, Hsu BR, Lin RH. 2002. The roles of interleukin-1 and interleukin-1 receptor antagonist in antigen-specific immune responses. *J Biomed Sci.* **9**: 26-33.

Ling S, Kuah MK, Sifzizul TTM, Kolkovski S, and Chong SA. 2006. Effect of dietary HUFA on reproductive performance, tissue fatty acid profile and desaturase and elongase mRNAs in female swordtail Xiphophorus helleri. *Aquaculture.* **241:** 204-214.

Liu BH, Kuo CF, Wang YC, and Ding ST. 2005. Effect of docosahexaenoic acid and arachidonic acid on the expression of adipocyte determination and differentiation-dependent factor 1 in differentiating porcine adipocytes. *J. Anim. Sci.* **83:**1516-1525.

Luciano AS, Cleber AP, Paulo CL, Talita T, Claudio TD, Felipe DP, and Ricardo AP. 2010. Vitamin E supplementation decreases muscular and oxidative damage but not inflammatory response induced by eccentric contraction. J Physiol Sci. **60:** 51–57.

Maachi M, Pieroni L, Bruckert E, Jardel C, Fellahi S, Hainque B, Capeau J, and Bastard JP. 2004. Systemic low-grade inflammation is related to both circulating and adipose tissue TNF-alpha leptin and IL-6 levels in women. *International Journal of obesity.* **28:** 993-997.

Maccarrone M, Meloni C, Manca-di-Villahermosa S, Cococcetta N, Casciani CU, Finazzi-Agrò A, and Taccone-Gallucci M. 2001. Vitamin E suppresses 5-lipoxygenase-mediated oxidative stress in peripheral blood mononuclear cells of hemodialysis patients regardless of administration route. *Am. J. Kidney Dis.* **37(5):** 964-969.

Maccarrone M, Taccone-Gallucci M, and Finazzi-Agrò A. 2003. 5-Lipoxygenase-mediated mitochondrial damage and apoptosis of mononuclear cells in ESRD patients. *Kidney International.* ***63:*** *33–36.*

MacKenzie SA, Roher N, Boltaña S, and Goetz FW. 2010. Peptidoglycan, not endotoxin, is the key mediator of cytokine gene expression induced in rainbow trout macrophages by crude LPS. *Mol Immunol.* **47(7-8):**1450-1457.

Madsen L, Petersen RK, and Kristiansen K. 2005. Regulation of adipocytes differentiation and function by polyunsaturated fatty acids. *Biochimica Acta.* **1740:** 266-286.

Maren M, Sigbjørn L, Christian B, Jakob H, Henrik H, Ingunn B, Theo HE M, and Eli G. 2008. Gene expression profiles in liver of pigs with extreme high and low levels of androstenone. *BMC Veterinary Research.* **4**: 29.

Mater MK, Thelen AP, and Jump DB. 1999. Arachidonic acid and regulation of hepatic lipogenic gene expression. *J. Lipid Res.* ***40:****1045–*1052.

Mehrabian M, Schulthess FT, Nebohacova M, Castellani LW, Zhou Z, Hartiala J, Oberholzer J, Lusis AJ, Maedler K, and Allayee H. 2008. Identification of ALOX5 as a gene regulating adiposity and pancreatic function. *Diabetologia.* **51(6):** 978-988.

Mehta NM, and Reilly MP. 2004. Mechanisms of the metabolic syndrome. *Drug discovery today: Disease Mechanisms.* **1:** 187-194.

Meydani M, Fielding RA, and Fotouhi N. 1996. Vitamin E. In: Sports Nutrition Vitamins and Trace Minerals. *Edited by Ira Wolinsky and Judy A. Driskell.* CRC Press. New York. 119-131.

Michael WK, 2011. Fatty Acid Synthesis. Available at http://themedicalbioche mistrypage.org/ lipid-synthesis.

Minami N, Ishimura S, Sakamoto E, Takishita K, Mawatri K, Okada, and Nakaya Y. 2002. Effect of eicosapentaenoic acid ethyl ester v. oleic acid-rich safflower oil on insulin resistance in type 2 diabetic model rats with hypertriacylglycerolaemia. *Br. J. Nutr.* **87:** 157–162.

Monahan FJ, Buckley DJ, Morrissey PA, Lynch PB, and Gray JI. 1992. Influence of dietary fat and R-tocopherol supplementation on lipid oxidation in pork. *Meat Sci.* **31:** 229-241.

Morera D, and MacKenzie S A. 2011. Is there a direct role for erythrocytes in the immune response? *Vet Res.* **42(1):** 89.

Mori Y, Hiraki Y, Shukunami C, Kakudo S, Shiokawa M, and Kagoshima M. et al. Stimulation of osteoblast proliferation by the cartilage-derived growth promoting factors chondromodulin-I and -II. *FEBS Letters.* **406:** 310–314.

Mori Y, Hiraki Y, Shukunami C, Kakudo S, Shiokawa M, Kagoshima M, Mano H, Hakeda Y, Kurokawa T, Suzuki F, and Kumegawa M. 1997. Stimulation of osteoblast proliferation by the cartilage-derived growth promoting factors chondromodulin-I and -II. *FEBS Lett.* **406(3):** 310-314.

Morley JW, Buckel JA, and Lankford TE Jr. 2007. Winter energy storage dynamics and cohort structure of young-of-theyear bluefish *Pomatomus saltatrix* of North Carolina. *Mar Ecol Prog Ser.* **334:** 273–286.

Mourente G, Diaz-Salvago E, Bell JG, and Tocher RD. 2002. Increased activities of hepatic antioxidant defence enzymes in juvenile gilthead sea bream (*Sparus aurata* L.) fed dietary oxidised oil: attenuation by dietary vitamin E. *Aquaculture.* **214:** 343-361.

Mowen K, and David M. 2000. Regulation of STAT1 nuclear export by Jak1. *Molecular and Cellular Biology.* **20:** 7273-7281.

Murashita K, Uji S, Yamamoto T, Ronnestad I, and Kurokawa T. 2008. Production of recombinant leptin and its effects on food intake in rainbow trout. *Comparative Biochemistry and Physiology B-Biochemistry & molecular biology.* **162:** 307-312.

Nicole AD, and Perry EB. 2008. Lipid Droplets in Lipogenesis and Lipolysis. *Endocrinology.* **17:** 68.

Novakofski J. 2004. Adipogenesis: Usefulness of in vitro and in vivo experimental models. *J. Anim. Sci.* **82:** 905–915.

O'Keefe TM, and Noble RL, Storage stability of channel catfish (*Ictalurus punctatus*) in relation to dietary level of α-tocopherol. *J. Fish. Res. Board Can.* **35:** 457–460.

Østbye TK, Kjær MA, Rørå AMB,Torstensen B, and Ruyter B. 2009. High n-3 HUFA levels in the diet of Atlantic salmon affect muscle and mitochondrial membrane lipids and their susceptibility to oxidative stress. In, **Doi:** *10.1111/j.1365-2095.2009.00721.x ed.*

Packer L. 1991. Protective role of vitamin E in biological systems. *Am. J. Clin. Nutr.* **53:**1050S-1055S.

Pallu R, Krishna M, and Channa C. 2001. Inhibition of 5-lipoxygenase by vitamin E. 2001. *FEBS Lett.* **139:** 39-43.

Palozza P, and Krinky NI. 1992. Beta-carotene and alpha-tocopherol are synergetic antioxidants. *Archives of Biochemistry and Biophysics.* **297:**184–187.

Pan DA, Lillioja S, Milner MR, Kriketos AD, Baur LA, Bogardus C, and Storlien, LH. 1995. Skeletal muscle membrane lipid composition is related to adiposity and insulin action. *Journal of Clinical Investigation.* **96:** 2802–2808.

Parola M, Muraca R, and Dianzani I. 1992. Vitamin E dietary supplementation inhibits transforming growth factor beta 1 gene expression in the rat liver. *FEBS Lett.* **308**: 267–270.

Parrish CC, Pathy DA, and Angel A. 1991. Dietary fish oils limits adipose-tissue hypertrophy in rats. *Metabolism-clinical and experimental.* **39:** 217-219.

Puigserver P, Wu Z, Park CW, Graves R, Wright M, and Spiegelman BM. 1998. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* **92:** 829–839.

Qiao L, Dai Y, Gu Q, Chan KW, Zou B, Ma J, Wang J, Hui Y, Lan, and Wong BCY. 2008. Down-regulation of X-linked inhibitor of apoptosis synergistically enhanced peroxisome proliferator-activated receptor γ ligand-induced growth inhibition in colon cancer. *Mol Cancer.* **7:** *2203.*

Qin QW, Ototake M, Noguchi K, Soma GI, Yokomizo Y, and Nakanishi T. 2001. Tumor necrosis factor alpha (TNF-α) like factor produced by macrophages in rainbow trout, *Oncorhynchus mykiss. Fish and Shellfish Immunology.* **11:** 245–256.

Rodríguez-Calvo R, Serrano L, Coll T, Moullan N, Sánchez RM, Merlos M, Palomer X, Laguna JC, Michalik L,Wahli W, and Vázquez-Carrera M. 2008. Activation of peroxisome proliferator-activated receptor beta/delta inhibits lipopolysaccharideinduced cytokine production in adipocytes by lowering nuclear factor-kappaB activity via extracellular signal-related kinase 1/2. *Diabetes.* **57(8):** 2149–2157.

Roux-Lombard P, Modoux C, and Dayer JM. 1989. Production of interleukin-1 (IL-1) and a specific IL-1 inhibitor during human monocyte-macrophage differentiation: influence of GM-CSF. *Cytokine.* **1:** 45–51.

Rubin P, and Mollison KW. 2007. Pharmacotherapy of diseases mediated by 5-lipoxygenase pathway eicosanoids. *Prostaglandins Other Lipid Mediat.* **83**:188–97.

Ruzickova J, Rossmeisl M, and Prazak T. 2004. Omega-3 PUFA of marine origin limit diet-induced obesity in mice by reducing cellularity of adipose tissue. *Lipids.* **39:** 1177–1185.

Ruzickova J, Rossmeisl M, Prazak T, Flachs P, Sponarova J, Vecka M, Tvrzicka E, Bryhn M, and Kopecky J. 2004. Omega-3 PUFA of marine origin limit diet-induced obesity in mice by reducing cellarity of adipose tissue. *Lipids.* **39:** 1177-1185.

Saeji JP, Verburg-van Kemenade LB, van Muiswinkel WB, and Wiegertjes GF. 2003. Daily handling stress reduces resistance of carp to *Trypanoplasma borreli*: *in vitro* modulatory effects of cortisol on leukocyte function and apoptosis. *Developmental and Comparative Immunology.* **27:** 233–245.

Sau SK, Paul BN, Mohanta KN, and Mohanty SN. 2004. Dietary vitamin E requirement, fish performance and carcass composition of rohu, Labeo rohita fry. *Aquaculture.* **240:** 359-368.

Scaccini, Nardini M, Aqino MD, Gentili V, Felice MDi, and Tomassi A. 1992. Effect of dietary oils on lipid peroxidation and on antioxidant parameters of rat plasma and lipoprotein fractions. *Journal Lipid Research.* **33:** 627–633.

Schaeffler A, Gross P, Buettner R, Bollheimer C, Buechler C, Neumeier M, Kopp A, Schoelmerich J, and Falk W. 2009. Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-κB pathway in adipocytes links nutritional signalling with innate immunity. *Immunology.* **126:** *233–*245.

Schaffer JE. 2003. Lipotoxicity: when tissues overeat. *Curr Opin Lipidol.* **14:** 281–287.

Schenk S, Saberi M, and Olefsky JM. 2008. Insulin sensitivity: modulation by nutrients and inflammation. *Journal of Clinical Investigation.* **118:** 2992–3002.

Scott MG, Bryan HB, James IC, Sidney CM, and Claude M. 2004. Report of the National Heart, Lung, and Blood Institute/American Heart Association Conference on Scientific Issues Related to Definition. *NHLBI/AHA Conference Proceedings.* **109:** 433-438.

Semb H, Peterson J, Tavernier J, and Olivecrona T. 1987. Multiple effects of tumor necrosis factor on lipoprotein lipase *in vivo*. *Journal of Biological Chemistry.* **262:** 8390–8394.

Semple RK , Crowley VC , Sewter CP , Laudes M, Christodoulides C, Considine RV, Vidal-Puig A, and O'Rahilly S. 2004. Expression of the thermogenic nuclear hormone receptor coactivator PGC-1is reduced in the adipose tissue of morbidly obese subjects. *International Journal of Obesity. **28:** 176–179.*

Serbinova EA, and Packer L. 1994. Antioxidant properties of alpha-tocopherol and alpha-tocotrienol, *Oxygen radicals in biological system, Pt D. Academic Press. San Diego.* **4495:** 354–366.

Sharma A, Belna J, Logan J, and Jean Hurteau. *2005* . Omega-3 fatty acids induce TGF-ß1 expression in epithelial ovarian cancer. *AACR Meeting Abstracts. **1:** 1432.*

Sheridan MA. 1998. Lipid dynamics in fish-aspects of absorption, transportation, deposition and mobilization. Comparative biochemistry and physiology B-*Biochemistry & molecular biology.* **90:** 679-690.

Shigeyuki T, Yuko Y, Saho M, Osamu N, Koji M, and Tasuku W. 2008. Isolation of epidermal cells and cDNA cloning of TNFdecoy receptor 3 of conger eel, *Conger myriaster*. *Fish Shellfish Immunol.* **24:** 366-371.

Shimomura Y, Tamura Y. and Suzuki M. 1990. Less body fat accumulation in rats fed a safflower oil diet than in rats fed a beef tallow diet. *J. Nutr.* **120:** 1291–1296.

Sierra S, Lara-Villoslada F, Olivares M, Jiménez J, Boza J, Xaus J. 2004. IL-10 expression is involved in the regulation of the immune response by omega 3 fatty acids. *Nutr Hosp.* **19**: 376-382.

Siiteri PK. 1987. Adipose tissue as a source of hormones. *Am J Clin Nutr.* **45:** 277-

Simopoulos AP. 2006. Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases. *Biomedicine & Pharmacotherapy.* **60:** 502–507

Skugor S, Skugor A, Todorcevic M, Torgersen J, Ruyter B, and Krasnov A. 2010. Exposure to lipopolysaccharide induces immune genes in cultured preadipocytes of Atlantic salmon. *Fish & Shellfish Immunology.* **29:** 817-824.

Skuladottir IH, Petursdottir DH, and Hardardottir I. 2007. The effects of omega-3 polyunsaturated fatty acids on TNF-alpha and IL-10 secretion by murine peritoneal cells in vitro. *Lipids.* **42(8):** 699-706.

Skuladottir IH, Petursdottir DH, and Hardardottir I. 2007. The effects of omega-3 polyunsaturated fatty acids on TNF-*a* and IL-10 secretion by murine peritoneal cells in vitro. *Lipids.* **42(8):** 699-706.

Stéphan G, *and* Lamour F. 1993. Incidence d'une supplémentation de l'aliment en acétate d'alpha-tocophérol chez le bar *Dicentrarchus labrax*, sur le taux d'alpha-tocophérol et sur la stabilité des lipides musculaires, in vivo et après stockage au congélateur à–20°C. *Aquaculture* **92:** 347–357.

Stylianou E, O'Neill, LAJ, Edbrooke MR, Woo P, and Saklatvala J. 1992. Interleukin-1 induces NFB through its type I and not type II receptor. *J. Biol. Chem.* **270:** 13757–13765.

Subir KD, Shkhes M, Geetanjali G, Rao DN, and Vasudevan DM. 2009. Protective effect of resveratrol and viatamin E against ethanol-induced oxidative damage in mice: Biochemical and immunological basis. *Indian Jouranal of biochemistry & Biophysics.* **47:** 32-37.

Suzuki Y, Jiang LL, Souri M, Miyazawa S, Fukuda S, Zhang Z, Une M, Shimozawa N, Kondo N, Orii T, and Hashimoto T. 1997. D-3-hydroxyacyl-CoA dehydratase:D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein deficiency: a newly identified peroxisomal disorder. *Am. J. Hum. Genet.* **61:** 1153–1162.

Swamy MV, Citineni B, Patlolla JM, Mohammed A, Zhang Y, and Rao CV. 2008. Prevention and treatment of pancreatic cancer by curcumin in combination with omega-3 fatty acids. *Nutr Cancer.* **1:** 81-89.

Taccone-Gallucci M, Manca-di-Villahermosa S, Battistini L, Stuffler RG, Tedesco M, and Maccarrone M. 2006. N-3 PUFAs reduce oxidative stress in ESRD patients on maintenance HD by inhibiting 5-lipoxygenase activity. *Kidney International.* **69:** 1450–1454.

Taccone-Gallucci M, Manca-di-Villahermosa S, Battistini L, Stuffler RG, Tedesco M, and Maccarrone M. 2006. N-3 PUFAs reduce oxidative stress in ESRD patients on maintenance HD by inhibiting 5-lipoxygenase activity. *Kidney Int.* **69(8):**1450-4.

Taniguchi T, and Yamamoto K. 2005. Anti-inflammatory effects of intravenous anesthetics on endotoxemia. *Mini Reviews in Medicinal Chemistry.* **5:** 241–245.

Thomsen C, Becker U, Winkler K, Christoffersen P, Jensen M, and Henriksen O. 1994. Quantification of liver fat using magnetic resonance spectroscopy. *Magn Reson Imaging.* **12:** 487–495.

Tiidus PM, and Houston ME. 1995. Vitamin E Status and Response to Exercise Training. *Sports Med.* **20:** 12-23.

Tilg H, and Moschen AR. 2006. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat. Rev. Immunol.* **6**: 772–783.

Todorcevic M, Kjær MA, Djaković N, Vegusdal A, Bente ET, and Ruyter B. 2008b. N-3 HUFAs affect fat deposition, susceptibility to oxidative stress, and apoptosis in Atlantic salmon visceral adipose tissue . *Comparative Biochemistry and Physiology.* **152:** 135–143.

Todorcevic M, Skugor S, Krasnov A, and Ruyter B. 2010a. Gene expression profiles in Atlantic salmonadipose-derived stromo-vascular fraction during differentiation into adipocytes.*BMC Genomics.* **11:** 39.

Todorcevic M, Skugor S, and Ruyter B. 2010b. Alterations in oxidative stress status modulate terminal differentiation in Atlantic salmon adipocytes cultivated in media rich in n−3 fatty acids. *Comparative Biochemistry and Physiology.* 156:309–318.

Todorcevic M, Vegusdal A, Gjøen T, Sundvold H, Torstensen BE, Kjaer MA, and Ruyter B. 2008a. Changes in fatty acids metabolism during differentiation of Atlantic salmon preadipocytes; effects of n-3 and n-9 fatty acids. *Biochim. Biophys. Acta.* **1781:** 326-335.

Tomonaga I, Jifeng Z, Kai C, Yusen L, Francisco S, Paul B, Bruce F, Yuqing E, and Taixing C. 2008. Nitroalkenes Suppress Lipopolysaccharide-Induced Signal Transducer and Activator of Transcription Signaling in Macrophages: A Critical Role of Mitogen- Activated Protein Kinase Phosphatase 1. *Endocrinology.* **149:** 4086-4094.

Tontonoz P, Hu E, Graves RA, Budavari AI, and Spiegelman BM. 1994. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. *Genes Devel.* **8:**1224–1234.

Traber MG, Baltimore MD, Williams, and Wilkins. 1999. Vitamin E. In: Ross AC, ed. *Modern nutrition in health and disease.* 347–62.

Traber MG, Burton GW, Ingold KU, and Kayden HJ. 1990. RRR- and SRR-alpha-tocopherols are secreted without dis-crimination in human chylomicrons, but RRR-alpha-toco-pherol is preferentially secreted in very low density-lipoproteins. *J. Lipid Res.* **31:** 675-685.

Trayhurn P, and Wood SI. 2004. Adipokines: inflammation and the pleiotropic role of white adipose tissue. *British Journal of Nutrition.* **92:** 347–355.

Tulic MK, Hodder M, and Forsberg A. 2010. Differences in innate immune function between allergic and nonallergic children: New insights into immune ontogeny. *J. Allergy Clin Immunol.* **127:** 470–8.

Tulic MK, Hodder M, Forsberg A, Mccarthy S, Richman T, D'vaz N, Van D B, Thornton Ca, and Prescott SL. 2010. Differences in innate immune function between allergic and

nonallergic children: New insights into immune ontogeny. *Journal of Allergy and Clinical Immunology.* **10:**1038.

Ulaganathan M, Jyotirmoi A, Geeta DL, Surendra KS, Amit KD, and Balaram G. 2009. Effects of vitamin E on mitochondrial dysfunction and asthma features in an experimental allergic murine model. *J Appl Physiol.* **107:** 1285–1292.

Upritchard JE, Sutherland WH, and Mann JI. 2000. Effect of supplementation with tomato juice, vitamin E, and vitamin C on LDL oxidation and products of inflammatory activity in type 2 diabetes. *Diabetes Care.* **23:** 733–738.

Uto-Kondo H, Reiko O, Chikako K,Yoshimi K, Hisako S, Osamu I, and Kazuo K. 2009.Tocotrienol Suppresses Adipocyte Differentiation and Akt Phosphorylation in 3T3-L1 Preadipocytes. *J. Nutr.* **139:** 51–57.

Vagusdal A, Sundvold H, Gjoen T, Ruyter B. 2003. An in vitro method for studying the proliferation and differentiation of Atlantic salmon preadipocytes. *Lipids.* **38:** 289-296.

Valk EEJ, and Hornstra G. 2000. Relationship between vitamin E requirement and polyunsaturated fatty acid intake in man: a review. *Int. J. Vitam. Nutr. Res.* **70:** 31–42.

Venkatraman JT, and 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(6):** 602-613.

Wakil SJ. 1989. Fatty acid synthethase, a proficient multifunctional enzyme. *Biochemistry.* **28:** *4523*-4530.

Waldman E, Lu SX, Hubbard VM. 2006. Absence of beta7 integrin results in less graft-versus-host disease because of decreased homing of alloreactive T cells to intestine. *Blood.* **107:** 1703–1711.

Wang H, Storlien LH, and Huang XF. 2002. Effects of dietary fat types on body fatness, leptin, and ARC leptin receptor, NPY, and AgRP mRNA expression. *Am. J. Physiol, Endocrinol Metabol.* **282:** E1352–E1359.

Wang J, Zhang H, Ma H, Lu B, Wang J, Li Y, and Li J. 2007. Inhibitory effect of dietary n-3 polyunsaturated fatty acids to intestinal IL-15 expression is associated with reduction of

TCRalphabeta+CD8 alpha+CD8 beta-intestinal intraepithelial lymphocytes. *J. Nutr Biochem.* **19(7):** 475-781.

Wang T, Huang W, Costa MM, Martin SA, and Secombes CJ. 2011. Two copies of the genes encoding the subunits of putative interleukin (IL)-4/IL-13 receptors, IL-4Rα, IL-13Rα1 and IL-13Rα2, have been identified in rainbow trout (Oncorhynchus mykiss) and have complex patterns of expression and modulation. *Immunogenetics.* **63(4):** 235-53.

Watanabe T, Takeuchi T, Wada M, and Uehara R. 1981. The relationship between dietary lipid levels and α-tocopherol requirement of rainbow trout. *Bull. Jpn. Soc. Sci. Fish.* **47:** 1463–1471.

Watanabe T. 1982. Lipid nutrition in fish. *Comparative biochemistry and physiology.* **73B:** 3-15.

Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, and Ferrante AW. 2003. Obesity is associated with macrophage accumulation in adipose tissue. *Journal of clinical investigation.* **112:** 1796-1808.

William CP, and Jaswinder KS. 2008. TNF-alpha and adipocyte biology. *The FEBS journal.* **582:** 117-13.

Williams ML, Coleman RA, Placezk, D, and Grunfeld C. 1991.*Biochim. Biophys. Acta.* **1096:** 162–169.

Witting LA, and Horwitt MK. 1964. Effect of Degree of Fatty Acid Unsaturation in Tocopherol Deficiency-Induced Creatinuria. *J. Nutr.* **82:** 19–33.

Witting PK, Petersson K, Letters J, and Stocker R. 1994. Anti-atherogenic effect of coenzyme Q(10) in apolipoprotein E gene knockout mice. *Free Radic. Biol. Med.* **29:** 295–305.

Wolfrum C, Borrmann CM, Borchers T, and Spener F. 2001. Fatty acids and hypolipidemic drugs regulate peroxisome proliferator- activated receptors α- and γ-mediated gene expression via liver fatty acid binding protein: A signaling path to the nucleus. *Proc. Natl. Acad. Sci.* **98:** 2323–2328.

Worgall TS, Sturley SL, Seo T, Osborne TF, and Deckelbaum RJ. 1998. Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element binding protein. *J. Biol. Chem.* **273:** 25537–25540.

Xu H, Barnes G, Yang Q, Tan G, Yang D, Chou C, Sole J, Nichols A, Ross J, and Tartaglia L, et al. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest.* **112:** 1821–1830.

Xu J, Nakamura MT, Cho HP, and Clarke SD. 1999. Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. *J. Biol. Chem.* **274:** 23577–23583.

Yasuhiro S, Kiyoshi M, Yoshimitsu A, and Takahide M. 2002. IL-1β production and gene expression by peptidoglycan from *Lactobacillus casei* in human dental pulp cells of deciduous teeth and permanent teeth. *International Journal of Oral-Medical Sciences.* **1:**130-138.

Zhang H, Halbleib M, Ahmad F, Manganiello VC, and Greenberg AS. 2002. Tumor necrosis factor-α stimulates lipolysis in differentiated human adipocytes through activation of extracellular signal-related kinase and elevation of intracellular CAMP. *Diabetes.* **51:** 2929–2935.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, and Friedman JM. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature.* **372:** 425-432.