

OMEGA-3 FATTY ACIDS, OBESITY-RELATED DISEASES, AND ADIPOKINES IN DOGS
AND CATS

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ABSTRACT

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The incidence of obesity in dogs and cats is increasing in recent years. In humans, obesity has been associated with the metabolic syndrome, which consists of a cluster of disorders including, insulin resistance, type II diabetes mellitus, hyperlipidemia, hepatic steatosis, hypertension, and atherosclerosis. Although naturally-occurring metabolic syndrome has not been described in dogs or cats, some components of this syndrome occur in diseases related to obesity, either as a predisposing factor (i.e. feline hepatic lipidosis and feline diabetes mellitus), a complicating factor (i.e. canine diabetes mellitus), or a consequence (i.e. canine hypothyroidism). Decreased adiponectin concentration has been implicated in the pathogenesis of the metabolic syndrome in humans. Findings regarding leptin are less consistent and either leptin deficiency or resistance was suggested. Consumption of n3-polyunsaturated fatty acids (PUFAs) has beneficial effects on various metabolic alterations of the syndrome. The present studies aimed to determine alterations in adipokines concentrations in obesity-related diseases in dogs and cats and to determine the effect of n3PUFAs on adipokines in health and disease.

In healthy dogs, circulating concentrations of docosapentaenoic acid were positively associated with concentrations of adiponectin and leptin; dietary fish oil supplementation for 30 days yielded an increase in adiponectin. In healthy cats, associations of n3PUFAs with adipokines were differential with body condition. Concentrations of eicosapentaenoic acid and

docosahexaenoic acid were positively associated with adiponectin concentrations in obese cats; while in non-obese cats, concentrations of eicosapentaenoic acid were negatively associated with concentrations of adiponectin and positively associated with concentrations of leptin.

In canine adipose tissue culture, eicosapentaenoic acid stimulated adiponectin secretion by mature adipocytes and inhibited interleukin-6 secretion by stromovascular cells. In contrast, palmitic acid inhibited adiponectin secretion by mature adipocytes and stimulated interleukin-6 secretion by stromovascular cells. In feline adipose tissue culture, arachidonic acid stimulated interleukin-6 secretion by stromovascular cells. Therefore, the stimulatory effect of n3PUFAs on adiponectin may be direct or mediated through inhibition of IL6. Moreover, these effects may be conveyed by n3PUFAs directly or by substitution of n6PUFAs or a saturated fatty acid.

Feline hepatic lipidosis was associated with hyperadiponectinemia, which is likely related to liver injury, and with hyperleptinemia, which is suggested to be specific to lipidosis. Canine hypothyroidism was associated with hyperleptinemia, which was associated thyroid hormone deficiency, and with hyperadiponectinemia, suggestive of adiponectin resistance. Diabetes mellitus was associated with hyperleptinemia in cats and hypoleptinemia in dogs, consistent with the opposite alterations in insulin secretion in these diseases.

In healthy animals, docosapentaenoic acid (dogs) or eicosapentaenoic acid (obese cats) was negatively associated with concentrations of triglyceride, supporting a hypolipidemic effect n3PUFAs. A negative association between n3PUFA and triglyceride was present in cats with hepatic lipidosis, but not the other diseases studied, suggesting preservation of a hypolipidemic effect of n3PUFA in overt feline hepatic lipidosis. Eicosapentaenoic was negatively associated with insulin concentrations in obese cats, supporting an insulin-sensitizing effect.

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid
ALA	Alpha-Linolenic Acid
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
BCS	Body Condition Score
%BF	Percent Body Fat
BSA	Bovine Serum Albumin
BUN	Blood Urea Nitrogen
CI	Confidence Interval
CV	Coefficient of Variation
DHA	Docosahexaenoic Acid
DKA	Diabetic Ketoacidosis
DM	Diabetes Mellitus
DPA	Docosapentaenoic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
EPA	Eicosapentaenoic Acid
FA	Fatty Acid
GEE	Generalized Estimating Equations

GLM	General Linear Model
GLUT	Glucose Transporter
HBSS	Hanks' Balanced Salt Solution
HCl	Hydrochloric Acid
HDL	High Density Lipoproteins
HL	Hepatic lipidosi
HMW	High molecular weight
HRP	Horseradish Peroxidase
IL	Interleukin
LDL	Low Density Lipoproteins
LMW	Low molecular weight
MUFA	Monounsaturated Fatty Acids
NAFLD	Non-Alcoholic Fatty Liver Disease
NSB	Non-specific Binding
PA	Palmitic Acid
PAV	Packed Adipocytes Volume
PBS	Phosphate Buffer Solution
PPAR	Peroxisome Proliferator-activated receptor
PUFA	Polyunsaturated Fatty Acid
QC	Quality Control
RIA	Radioimmunoassay
SD	Standard Deviation
SE	Standard Error

SFA	Saturated Fatty Acid
SVC	Stromovascular Cell
Spl	Sample
St	Standard
TNF	Tumor Necrosis Factor
T3	Triiodothyronine
T4	Thyroxine
TC	Total Count
TMB	3, 3', 5, 5'-Tetramethylbenzidine
UDM	Uncomplicated Diabetes Mellitus
VLDL	Very Low Density Lipoproteins

CHAPTER 1

ADIPOKINES AND N3PUFAS IN HEALTHY DOGS AND CATS

1.1. Introduction

1.1.1. *Obesity, adipose tissue, and adipokines*

Obesity is defined as the accumulation of excessive amounts of adipose tissue in the body that adversely affects health (Kopelman, 2000). Similar to the well-established phenomenon in the human population of Western countries, the incidence of obesity in dogs and cats is increasing in recent years, reaching about 30% of the pet population (Lund et al., 2005, 2006). A recent study reported reduced lifespan in obese human subjects (Whitlock et al., 2009), and in dogs, life-long ad-libitum feeding was demonstrated to shorten life span compared to energy restriction (Kealy et al., 2002).

Obese humans are at increased risk to develop the metabolic syndrome, which consists of a cluster of disorders including, insulin resistance and type II diabetes mellitus (DM), hyperlipidemia, hypertension, atherosclerosis and coronary heart disease, and hepatic steatosis. Obese humans are also more likely to suffer from certain cancers, osteoarthritis, and respiratory disorders (Kopelman, 2000). Similarly, obesity has been associated with DM, orthopedic and urinary tract diseases, and increased risk for neoplastic diseases in dogs and cats, as well as hepatic lipidosis (HL) in cats and hypothyroidism and hyperadrenocorticism in dogs (German, 2006). Profound research in human medicine in recent years focused on the mechanisms by which the enlarged adipose tissue mass that defines obesity leads to increased risk for certain diseases. This research provided considerable knowledge; nevertheless, many details of these mechanisms remain unknown. The occurrence of naturally-occurring metabolic syndrome has not been described in dogs or cats. Moreover, our knowledge regarding potential mechanisms linking disease to obesity in these species is scarce to date.

As the major site of energy storage, adipose tissue was traditionally considered to be a passive organ which responds to signals dictated by energy demands: it stores energy in the form of triglycerides during nutritional abundance and releases it as free FAs during nutritional deprivation. Over the last two decades, adipose tissue has been increasingly recognized as an important active endocrine organ participating in energy homeostasis by secretion of a wide range of hormones and protein factors, including adiponectin, leptin, Tumor necrosis factor (TNF)- α , and Interleukin (IL)-6, among others. These peptides are collectively termed “adipokines”, and have multiple effects on various metabolic pathways. Alterations in secretion and function of these adipokines in obesity may contribute to the development of obesity-related diseases.

Concurrently with the discovery of adipokines, it was also realized that adipose tissue is not a diffuse homogenic tissue, but a complex organ that contains a mixture of cell types and its composition and function vary among different anatomic locations. The major cell type of adipose tissue is the mature adipocyte, which is a large triglyceride-filled cell. Other cell types that account for up to 50% of the cells of adipose tissue include preadipocytes, fibroblasts, endothelial cells, undifferentiated mesenchymal cells, and macrophages (Fain et al., 2002; Fain, 2006). The presence of pre-adipocytes is crucial to the expansion of adipose tissue that occurs in obesity. These cells are recruited when existing adipocytes reach a critical level of hypertrophy, resulting in adipose tissue hyperplasia. Monocytes and macrophages in adipose tissue have been identified as important contributors to obesity-related as sources of proinflammatory cytokines and their numbers and activity increase as adipose tissue expands (Weisberg et al., 2003).

Research in humans and animal models has shown that adipose tissue in different anatomic locations have distinct biologic behavior due to local influences on gene expression

and differentiation. In humans, it is clear that the pathologic sequelae of obesity are influenced by the preferential deposition of fat into visceral rather than subcutaneous deposits.

Accordingly, the metabolic syndrome is associated with abdominal obesity specifically (Scaglione et al., 2010; Abrams and Levitt Katz, 2011). Compared with the subcutaneous depot, visceral fat is relatively resistant to the antilipolytic effects of insulin, which contributes to the increase in circulating free FA concentrations in subjects with abdominal obesity. Elevated free FAs can block insulin signaling in skeletal muscle and liver, leading to compensatory hyperinsulinemia (Zierath et al., 1998). In addition, differences in the secretion of adipokines among various adipose tissue depots may also play a role in the pathogenesis of the metabolic syndrome in subjects with abdominal obesity (Fried et al., 1998; Fisher et al., 2002; Fain et al., 2004b).

Adiponectin

Adiponectin is a 30-kDa adipocyte-derived plasma protein which was identified by four research groups independently in the mid-1990s and was named AdipoQ, apM1 - adipose most abundant gene transcript 1, GBP28 - gelatin-binding protein 28, or Acrp30 - adipocyte complement-related protein 30 (Scherer et al., 1995; Hu et al., 1996; Maeda et al., 1996; Nakano et al., 1996). Adiponectin was first discovered in association with its antiatherosclerotic effects in humans. And soon after was also found to have profound insulin sensitizing effect, and anti-inflammatory effects.

Human adiponectin is encoded by the ADIPOQ gene (previously named APM1 or ACDC), which spans 17 kb on chromosome locus 3q27 (Saito et al., 1999). Although the

ADIPOQ gene is expressed mainly in adipocytes, recent studies have found that adiponectin gene expression can be induced in hepatocytes (Yoda-Murakami et al., 2001), myotubes (Staiger et al., 2003), bone-forming cells (Berner et al., 2004), and cardiomyocytes (Pineiro et al., 2005). To date, adiponectin gene was generally reported to be expressed exclusively in adipose tissue in dogs (Eisele et al., 2005; Ishioka et al., 2006) and cats (Ishioka et al., 2009). A very low level of adiponectin expression was reported in skeletal muscle of cats in one study (Zini et al., 2009). Although regulation of adiponectin gene expression remains to be fully elucidated, it has been shown that Peroxisome Proliferator-activated receptor (PPAR)- γ , an important transcriptional activator of many adipocytes-specific genes, is required for adiponectin gene induction, and a functional PPAR-responsive element in the promoter region of the human adiponectin gene has been identified (Iwaki et al., 2003). Pharmacological activation by PPAR γ -agonists was demonstrated to increase adiponectin concentrations in dogs (Kasai et al., 2008) similar to findings in humans (Miyazaki et al., 2004; Riera-Guardia and Rothenbacher, 2008).

Adiponectin is the most abundant adipokine, circulating in relatively high concentrations compared to other adipokines, and comprising 0.01% of total serum proteins in humans. Secreted adiponectin consists of an N-terminal species-specific variable region followed by a conserved collagenous domain, and a C-terminal globular domain (Yamauchi et al., 2003a). In humans and mice, adiponectin is found in serum in a number of complexes which include trimers and hexamers, collectively described as low molecular weight (LMW) oligomers, and as multimeric forms of 12 to 18 subunits, called the high molecular weight (HMW) multimers (Scherer et al., 1995). The distribution of adiponectin multimers in cat has been recently shown to be comparable to that of humans (Tan et al., 2011). An 180 kd LMW oligomer of adiponectin was initially described in dogs' serum, but the HMW form could not be resolved under the

electrophoresis conditions applied in that study (Brunson et al., 2007). Both LMW and HMW multimers have been recently described in dogs' serum as well (Verkest et al., 2011b; Wakshlag et al., 2011)

Adiponectin has various effects on multiple body systems as evident from the presence of its receptors in different target tissues. Two types of receptors have been described; ADR1 is present in most tissues, it is highly preserved from yeast to mammals and has also been described in cats (Ishioka et al., 2009), and ADR2, which is most abundantly expressed in the liver in mice (Yamauchi et al., 2003a). It has been demonstrated that two types of adiponectin receptor have different binding affinity for globular and full-length adiponectin, ADR1 is a high-affinity receptor for globular adiponectin but a very low-affinity receptor for full-length adiponectin, whereas ADR2 is an intermediate affinity receptor for globular and full-length adiponectin. In vitro studies have revealed that both isoforms of adiponectin receptor can mediate increased AMP-activated protein kinase (AMPK) phosphorylation and PPAR α activity by adiponectin binding, thus activating FA oxidation and glucose uptake (Yamauchi et al., 2003a).

Effects of adiponectin on its target tissue

The main target organs of adiponectin are the skeletal muscle and liver, where it has potent effects on carbohydrate and lipid metabolism, leading to improved insulin sensitivity. In the skeletal muscle, adiponectin improves FA utilization, increasing their uptake and oxidation. The molecular mechanisms underlying these effects include up-regulation of several genes involved in muscle lipid metabolism, such as FA translocase (FAT/CD36), acyl-CoA oxidase (ACO), the rate-limiting enzyme of the β -oxidation pathway in peroxisomes, and mitochondrial uncoupling protein 2 (UCP2). In addition, adiponectin stimulates glucose transport into the

myocyte by enhancing the translocation of Glucose transporter (GLUT)-4 molecules to the cell membrane, and decreases glycogen synthesis by inhibition of glycogen synthase. In the liver, adiponectin decreases FA uptake and increases their oxidation, thereby reduces the hepatocyte triglyceride content. It also suppresses hepatic glucose production by down-regulation of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) gene expression (Karbowska and Kochan, 2006).

Adiponectin also has multiple anti-inflammatory effects. It has been shown to induce production of anti-inflammatory cytokines (IL10, IL1 receptor antagonist) by human monocytes, macrophages and dendritic cells on one hand, and to suppress macrophage production of pro-inflammatory cytokines (TNF α , IL6, interferon- γ) and mitigates their phagocytic activity in response to stimulation with lipopolysaccharide (LPS) on the other hand. Within the adaptive immune system, adiponectin abates T-cell ability to evoke an allogenic T-cell response and inhibits B-cell lymphopoiesis (Ouchi et al., 2000; Yokota et al., 2000; Yokota et al., 2003; Wolf et al., 2004).

In the vascular endothelium, adiponectin inhibits TNF α induced activation of the nuclear transcription factor, Nuclear Factor- κ B in endothelial cells, leading to decreased expression of adhesion molecules and inhibition of monocyte adhesion to endothelial cells. It also decreases expression of inflammatory cytokines and increases nitrous oxide production, inhibits smooth muscle cell proliferation, and suppresses macrophage to foam cell transformation by inhibiting cholesterol accumulation. These combined effects lead to a decreased tendency for atherosclerosis (Fantuzzi and Mazzone, 2007).

Factors determining adiponectin expression and secretion

Body fat content is considered the major determinant of circulating concentrations of adiponectin in humans and rodent models with a negative relationship between adiponectin and body fat (Arita et al., 1999; Kadowaki et al., 2003). Similarly, concentrations and adipose tissue expression of adiponectin were significantly lower in obese and overweight dogs compared to lean dogs and concentrations were demonstrated to decrease following induced weight gain (Ishioka et al., 2006; Gayet et al., 2007). Likewise in cats, circulating concentrations of adiponectin were significantly lower in obese compared to lean cats and increased following weight loss (Hoenig et al., 2007; Ishioka et al., 2009; Muranaka et al., 2011). In contrast, other studies reported no association between adiponectin and body condition in dogs (Mitsuhashi et al., 2010; Grant et al., 2011; Verkest et al., 2011a) and no change following weight loss in dogs (German et al., 2009; Wakshlag et al., 2011) or weight gain in cats (Tan et al., 2008). Potential suggested mechanisms leading to reduction of adiponectin expression and secretion in obesity include the decrease in expression of PPAR γ in obese adipose tissue that has been demonstrated in dogs (Gayet et al., 2007) and cats (Hoenig et al., 2008), similar to humans and rodent models (Lefebvre et al., 1998), and inhibition of adiponectin expression by inflammatory cytokines, which are secreted from the increasing numbers of macrophages and other cells in the obese adipose tissue (Weisberg et al., 2003). The absence of an association of body condition to adiponectin concentrations in some studies in dogs and cats remains unexplained.

Genetic factors may have some effect on adiponectin concentrations. In humans, several single nucleotide polymorphism as well as mutations in the adiponectin gene have been discovered and were associated with reduced adiponectin concentrations (Kadowaki and Yamauchi, 2005).

A gender effect on concentrations of adiponectin exists in humans; concentrations of adiponectin are higher in women than man both in lean and obese subjects (Arita et al., 1999; Nishizawa et al., 2002) and it was shown that the difference exists in the form of HMW, but not in LMW adiponectin (Xu et al., 2005). Testosterone treatment decreased circulating adiponectin in rodents and was demonstrated to decrease secretion of HMW adiponectin from adipocytes in vitro (Nishizawa et al., 2002; Xu et al., 2005). Studies in dog and cats to date did not reveal gender difference in adiponectin. This could potentially be explained by the inclusion of mostly neutered animal in some of the studies (Ishioka et al., 2006; Tan et al., 2011). In addition, a recent study reported no long-term change in serum concentrations of adiponectin in Beagle dogs following orchidectomy (Tvarijonaviciute et al., 2011).

Other hormones and cytokines have been shown to affect adiponectin expression. In murine adipocytes cell line, adiponectin gene expression was downregulated by insulin and dexamethasone (Fasshauer et al., 2002). In human adipose tissue it was shown to be up-regulated by insulin and Insulin-like growth factor 1 (Halleux et al., 2001). The clinical significance of these findings is not clear. Expression of adiponectin in human adipose tissue culture was also decreased by IL6 and TNF α (Bruun et al., 2003). In canine adipocytes differentiated in culture, expression of adiponectin was decreased by TNF α , but was not affected by dexamethasone (Ryan et al., 2010).

Studies in humans report conflicting results regarding the effect of feeding on concentrations of adiponectin. Some studies report increased (Musso et al., 2005), some report decreased (Esposito et al., 2003; Halberg et al., 2005; Stirban et al., 2007) concentrations, while others revealed no change (Peake et al., 2003; Imbeault et al., 2004; Shimabukuro et al., 2007) in concentrations of adiponectin following a meal. In mice, adiponectin concentrations have been

shown to be increased by fasting and reduced upon refeeding (Tsuchida et al., 2004). Similarly, a recent study in healthy lean cats reported a significant reduction from fasting to postprandial in total and HMW adiponectin, but not LMW adiponectin. These differences were present in cats that were fed a high carbohydrate diet but not in cats fed a low carbohydrate diet (Tan et al., 2011).

The effect of diet on adiponectin expression and circulating concentrations has been studied extensively in humans and rodent models. A carbohydrate-rich diet was associated with lower adiponectin concentrations in men (Pischon et al., 2005), but higher concentrations in rats (Kamari et al., 2007). In a recent study in lean healthy cats, feeding a high carbohydrate diet for 4 weeks was associated with increased total and LMW adiponectin but no change in HMW adiponectin, while a low carbohydrate diet was associated with no change in total adiponectin, but increased HMW and decreased LMW adiponectin (Tan et al., 2011). It was speculated that modulation of dietary carbohydrate intake results in alterations in adiponectin profiles as a result of changes in the production and clearance of adiponectin. More specifically, in order to maintain concentrations of the metabolically active HMW adiponectin in the face of increased carbohydrate intake, which promotes clearance of HMW adiponectin, production of total adiponectin is increased which results in a net increase in LMW and total adiponectin. In dogs, feeding low vs. high glycemic index starch and diacylglycerol vs. triacylglycerol did not have an effect on adiponectin concentrations in obese intact female Beagle dogs (Mitsuhashi et al., 2010). However, adiponectin multimers were not determined in that study.

Studies in human subjects reported a direct relationship between circulating n3PUFAs and adiponectin concentrations in human subjects (Fernandez-Real et al., 2005; Hernandez-Morante et al., 2007; Kim et al., 2010). Additional studies have demonstrated an increase in

adiponectin following dietary supplementations with long chain n3PUFAs in rodent models as well as human subjects (Rossi et al., 2005; Flachs et al., 2006; Neschen et al., 2006; Duda et al., 2007; Itoh et al., 2007; Higuchi et al., 2008), supporting a causal effect of these FAs on adiponectin secretion.

Dietary supplementation with green tea extract resulted in increased adiponectin concentration in obese dogs (Serisier et al., 2008).

Leptin

Leptin is a 16-kDa plasma protein synthesized mainly in adipose tissue. It is encoded by the obese (*lep*) gene that was identified in the mid 1990's as the causative mutation in ob/ob mice (Zhang et al., 1994). Leptin is an important regulator of adipose tissue mass and increases insulin sensitivity through multiple effects on satiety, energy expenditure, and neuroendocrine function. In leptin-sensitive lean individuals, short-term increases in circulating leptin in response to feeding promote satiety. However, with chronic overfeeding and obesity, resistance to leptin action develops. Since increased energy stores would favor survival in periods of famine, the adipostatic aspect of leptin action may have been selected against during the course of evolution. The development of leptin resistance limits the capacity of rising leptin to prevent obesity in most situations. Several mechanisms have been proposed. These include intravascular defects, defects in the transport of leptin into the central nervous system, a failure of leptin to activate the leptin receptor or post-receptor defects in the leptin signal transduction pathway (Kahn and Flier, 2000).

Leptin is primarily produced by adipose tissue, although it has been shown to be produced by other cells/organs such as bone marrow adipocytes, placenta, mammary gland, liver, and stomach (Smith-Kirwin et al., 1998; Reidy and Weber, 2000; Guilmeau et al., 2004). To date, expression of leptin have been documented exclusively in adipose tissue in dogs (Iwase et al., 2000; Eisele et al., 2005) and cats (Sasaki et al., 2001). Little is known about mechanisms regulating leptin gene transcription. In mature human adipocytes, PPAR γ agonists decrease leptin promoter activity and leptin production; however, no PPAR response element in the leptin promoter has yet been identified (Lee and Fried, 2009). In contrast, a PPAR γ agonist was demonstrated to increase leptin expression in canine adipocytes differentiated in culture (Ryan et al., 2010).

Leptin mediates its effects by binding to specific leptin receptors (ObRs) expressed in the brain as well as in peripheral tissues (Lee et al., 1996). Alternative splicing generates several isoforms of ObRs. The ObRa isoform (the short leptin receptor isoform) is thought to play an important role in transporting leptin across the blood–brain barrier. The ObRb isoform (the long leptin receptor isoform) mediates signal transduction and is strongly expressed in the hypothalamus, an important site for the regulation of energy homeostasis and neuroendocrine function. The binding of leptin to the ObRb receptor activates several signal transduction pathways, including Janus Kinase-Signal Transducer and Activator of Transcription-3 (JAK-STAT3), which is important for regulation of energy homeostasis, and Phosphatidylinositol 3-Kinase (PI3K), which is important for regulation of both food intake and glucose homeostasis. Other pathways, including Mitogen-activated Protein Kinase (MAPK), 5' Adenosine Monophosphate-activated Protein Kinase (AMPK), and the Mammalian Target of Rapamycin (mTOR), have been proposed to be downstream of leptin (Kelesidis et al., 2010). Leptin exerts

its effects predominantly through actions within the central nervous system and possibly by additional direct effects at the level of insulin target tissues.

Effects of leptin on its target tissues

Leptin appears to function within a long term control, influencing the quantity of food consumed relative to the amount of energy that is expended over time rather within a time frame of a single meal (Friedman, 2002). Leptin's effects as an adipostat and insulin-sensitizer are exerted predominantly through actions within the central nervous system. The hypothalamic pathways involved in these actions are incompletely understood, although a role for melanocortin signaling pathways has been suggested. These central effects may be transmitted to the periphery through a variety of mechanisms, including the effects of altered appetite to decrease nutrient flux into the body and the effects of leptin on neuroendocrine or neural pathways. Neuropeptide-Y and/or corticotrophin-releasing hormone have also been implicated as mediators of leptin's actions. Hypothalamic neuropeptide-Y is a potent stimulator of food intake and inhibitor of energy expenditure and leptin has been shown to decrease neuropeptide-Y synthesis and release in normal animals. Corticotropin-releasing hormone inhibits food intake and increases energy expenditure and leptin has been shown to act in the central nervous system to stimulate corticotrophin-releasing hormone gene expression (Kahn and Flier, 2000). .

In addition to the actions of leptin to modify metabolism via the brain, substantial data support the notion that leptin may have important effects through direct action on peripheral target tissues, including β -cells, liver, muscle, and, adipose tissue. Some of these effects promote insulin sensitivity, while others seem to antagonize it. In muscle and β -cells, leptin promotes lipid oxidation and inhibits lipid synthesis. Leptin also increases energy expenditure in brown

adipose tissue and perhaps in white adipose tissue and skeletal muscle through uncoupling mitochondrial oxidative metabolism. On the other hand, leptin has been shown to impair insulin stimulation of glucose transport, and inhibit glycogen synthase, lipogenesis, FA oxidation, and insulin binding to its receptors in isolated rat adipocytes and to attenuate some of insulin's signals and reduce suppression of gluconeogenesis in hepatocytes (Kahn and Flier, 2000).

Factors determining leptin expression and secretion

Body fat content is the major determinant of leptin expression and a direct relationship is reported in humans and rodents (Maffei et al., 1995) as well as dogs (Ishioka et al., 2002b; Ishioka et al., 2007) and cats (Appleton et al., 2000; Backus et al., 2000; Shibata et al., 2003). Moreover, concentrations of leptin were demonstrated to increase in dogs after induced weight gain (Ishioka et al., 2002b) and to decrease in cats following weight loss (Hoenig et al., 2007). Chronic elevations in concentrations of leptin in obese subjects result from a state of leptin resistance and inability of leptin to lead to a decrease in body fat mass.

A gender effect on serum concentrations of leptin is present in humans and rodents and concentrations of leptin are higher in females than males. Estradiol has been shown to increase leptin expression and concentrations (Shimizu et al., 1997), while a negative association of serum concentrations of leptin and testosterone was demonstrated (Behre et al., 1997). One study demonstrated higher leptin concentrations in premenopausal women than in men, and lower concentrations in postmenopausal women than in premenopausal women, but still higher than in men (Shimizu et al., 1997). Another study also documented higher leptin concentrations in women than men, but revealed that this gender difference was not apparently explained by sex hormones or body fat distribution and suggested that women may be resistant to leptin's putative

lipostatic actions (Saad et al., 1997). In cats, neutering with weight maintenance was shown to increase circulating concentrations of leptin in males but did not alter concentrations in females (Hoenig and Ferguson, 2002). Another study demonstrated a decrease in adipose tissue mRNA expression of leptin in female cats following neutering and food restriction to maintain body weight (Belsito et al., 2009). In dogs, no effect of gender on leptin concentrations was demonstrated (Ishioka et al., 2002b; Ishioka et al., 2007).

Other hormones affect expression and secretion of leptin. Glucocorticoids increase leptin mRNA levels in humans and rodents (Fried et al., 2000; Lee and Fried, 2009). Similarly, dexamethasone injection, alone or in conjunction with feeding, was demonstrated to increase leptin concentrations in dogs (Ishioka et al., 2002a; Nishii et al., 2006). Daily oral administration of prednisolone, however, did not affect plasma leptin concentrations in dogs (Nishii et al., 2006). This difference may be related to the relative dose of glucocorticoids. Dexamethasone has been shown to increase leptin expression in canine adipocytes differentiated in culture (Ryan et al., 2010), similar to findings in humans and rodents. In addition, insulin increases leptin synthesis and secretion in humans (Lee and Fried, 2009) and was shown to increase circulating concentrations of leptin in dogs (Ishioka et al., 2005). Administration of triiodothyronine (T3) to cats did not alter concentrations of leptin (Hoenig et al., 2008).

Feeding increases leptin concentrations in humans through increases in insulin, glucose, and amino acids. Conversely, a decline in energy or nutrient availability may contribute to the decreased leptin levels with fasting via a decrease in Mammalian Target of Rapamycin (mTOR) and increase in AMP-activated protein kinase (AMPK) signaling (Lee and Fried, 2009). Similarly, feeding was demonstrated to increase concentrations of leptin in dogs (Ishioka et al., 2005; Nishii et al., 2006). In cats, insulin secretion induced by feeding did not appear to affect

leptin secretion in overweight cats until 15–18 h after consumption of the meal and a relatively small meal stimulus caused longer post-prandial delay in leptin increase (Appleton et al., 2002). Another study in cats revealed relatively small increase in concentrations of leptin following feeding (Backus et al., 2000), and it was speculated that the difference from humans and rats is due to the fact that blood glucose concentration is not profoundly reduced by withholding of food in cats and that acetate is preferred to glucose in lipogenesis in cats, whereas glucose is the preferred substrate in humans and rats.

Dietary composition has been shown to affect leptin concentrations. In humans, carbohydrate-rich diet was demonstrated to increase leptin concentrations (Herrmann et al., 2001). In contrast, in cats no difference in leptin concentrations was found between cats fed a high fat and low carbohydrate diet and cats fed a low fat high carbohydrate diet (Hoenig et al., 2007).

Studies on the effect of n3PUFA on leptin report inconsistent results. In rats, supplementation of a high-fat diet with n3PUFA was demonstrated to decrease leptin expression in adipose tissue and increase the expression of its receptor in the liver in one study (Ukropec et al., 2003) but to increase leptin concentrations in another study (Peyron-Caso et al., 2002). In humans, increased leptin concentrations were associated with decreased n3PUFA concentrations in patients with acute myocardial infarction (Oda et al., 2005).

Pro-inflammatory cytokines: Tumor Necrosis Factor- α and Interleukin-6

During the past 15 years, it became clear that chronic low-grade inflammation is a key feature of obesity. This condition is principally triggered by nutrients and metabolic surplus, and

is characterized by abnormal cytokine production, increased acute-phase reactants and other mediators, and activation of a network of inflammatory signaling pathways, similar to those involved in classical inflammation. Nevertheless, the classic features of inflammation, namely swelling, redness, pain, and fever, have not been observed with obesity-related inflammation (Hotamisligil, 2006). In addition, obesity has been shown to be associated with infiltration of the adipose tissue with macrophages and the number of macrophages was found to directly correlate to body mass (Weisberg et al., 2003).

TNF α is a 26-kDa transmembrane protein, which is released into the circulation as a 17-kDa soluble protein after extracellular cleavage by a metalloproteinase. TNF α has two main receptors, which are expressed on many cells, including adipocytes. Although circulating TNF α concentrations are relatively low and have not been consistently associated with obesity, tissue expression levels of TNF α are directly related to body condition both in humans and rodent models (Hotamisligil et al., 1995; Yu and Ginsberg, 2005).

IL6 is a protein of 22 to 27 kDa, with various degrees of glycosylation. In contrast to TNF α , IL6 circulates at relatively high concentrations, and adipose tissue contributes about a third of the circulating IL6. Adipose tissue expression and serum concentrations of IL6 are directly related to body condition both in humans and rodent models (Vozarova et al., 2001; Yu and Ginsberg, 2005).

In dogs, TNF α concentrations were demonstrated to increase in response to overfeeding (Gayet et al., 2004) and to decrease with weight loss (German et al., 2009). Adipose tissue expression of TNF α was shown to be higher in obese than in lean cats (Miller et al., 1998; Hoenig et al., 2006), and expression of IL6 was demonstrated to increase following ad libitum feeding and weight gain (Belsito et al., 2009).

IL6 and TNF α display multiple effects on carbohydrate and lipid metabolism, leading to impairment of insulin sensitivity and lipid metabolism.

TNF α exerts its effects via paracrine effects on adipocytes and remote effects in the liver. In adipose tissue, TNF α promotes lipolysis, leading to elevation of circulating FAs concentrations by making triglycerides accessible to hormone sensitive lipase and through suppression of lipoprotein lipase. Additionally, TNF α reduces the expression of genes involved in adipogenesis and lipogenesis in adipocytes, likely through NF κ B-mediated transcription. In the liver, TNF α increases the expression of genes involved in de novo FA synthesis, while decreasing expression of those involved in FA oxidation, thereby stimulating very low density lipoproteins (VLDL) production. These effects of TNF α on adipose tissue and the liver lead to hypertriglyceridemia. TNF α also impairs insulin signaling by activating Ser/Thr kinases that act on insulin receptor and insulin-receptor substrate molecules, making them poor substrates for insulin-mediated tyrosine phosphorylation and signal propagation. In addition, TNF α decreases adiponectin and increases IL6 secretion (Feingold and Grunfeld, 1992; Hotamisligil, 1999; Yu and Ginsberg, 2005).

IL6 also has multiple effects on adipose tissue, including increased triglyceride lipolysis and suppression of lipoprotein lipase activity, as well as inhibition of adiponectin expression. In the liver, IL6 impairs insulin signaling by downregulation of insulin-receptor substrate and upregulation of suppressor of cytokine signaling 3, a negative regulator of insulin signaling (van Hall et al., 2003; Yu and Ginsberg, 2005).

1.1.2. Dietary FAs and beneficial effects of *n*3PUFAs

A fatty acid (FA) is a hydrocarbon chain with an even number of carbon atoms and its name is designated based on the number of carbons (X), the number of double bonds (Y), and the location of double bond closest to the methyl end (Z), i.e. CX:Yn-Z. The structure of the FA determines its functional and health effects. These FAs contribute to the variability in the form and effects of fats.

Almost all mammals, including dogs and cats, are able to synthesize saturated FAs (SFAs) de novo from glucose or amino acids via a common precursor, acetyl CoA. The products of this synthesis are 16- and 18-carbon SFAs. Triglycerides comprise the largest proportion of dietary fat and are composed of a short glycerol backbone attached to three FAs. SFAs, synthesized de novo or of dietary origin, can subsequently be desaturated to monounsaturated FAs (MUFAs). This process involves specific enzymes (Δ -x desaturase) that introduce a single double bond some distance (x) from the carboxyl carbon, i.e. insertion of a double bond between carbon atoms 9 and 10 is performed by Δ -9 desaturase, whereas insertion between carbon atoms 6 and 7 is performed by a Δ -6 desaturase. Enzymes regulating these reactions are active when high-carbohydrate, low-fat diets are fed but have low activity when high-fat diets are fed. Consequently, animals fed relatively high-fat diets will only synthesize limited amounts of the needed FAs. Instead, they directly use the dietary supply of FAs (Salati and Goodridge, 1996).

Mammals can only produce MUFA of the *n*7 and *n*9 FA families by use of Δ -6 or Δ -9 desaturases. In contrast, plants synthesize *n*6 and *n*3 FAs owing to the presence of additional desaturases, including a Δ -12 desaturase that generates linoleic acid (LA; C18:2*n*-6) from the monounsaturated precursor, oleic acid (OA; C18:1*n*-9), and a Δ -15 desaturase that inserts a

double bond into LA to generate α -linolenic acid (ALA; C18:3n-3). Furthermore, marine plants are able to insert additional double bonds into ALA and subsequently elongate the carbon chain, which accounts for the formation of the long-chain n3PUFAs and their abundance in marine plants. Terrestrial plants, however, do not analogously insert additional double bonds into LA. Thus, arachidonic acid (AA; C20:4n-6) is not found in plant tissues. Therefore, oils manufactured from terrestrial plants may be rich in either LA (Sunflower oil) or ALA (Flaxseed oil), while fish oil is rich in long-chain n3PUFAs, eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) (Cook, 1996).

Since mammals are incapable of synthesizing both n6 and n3 FAs, mammalian diets must include essential FAs, which at the minimum consist of LA and ALA. Once ingested, in most mammals these dietary essential FAs are converted to longer chain derivatives through regulation by Δ -6 desaturases. In cats, these conversions are greatly limited because of low Δ -6 desaturase activity (Rivers et al., 1975). Therefore, LA and ALA cannot be converted to other physiologically important long-chain PUFAs, such as AA, EPA, and DHA, to the extent necessary for certain life stages or processes. Thus, long-chain n6 and n3PUFA are considered essential in cats. Although adult dogs have substantial Δ -6 desaturase activity in general, there is evidence that long-chain n3PUFAs may also be conditionally essential for dogs (Bauer, 2006, 2007).

Docosapentaenoic acid (DPA; C22:5n-3) is suggested to serve as an important circulating or tissue reservoir for either EPA or DHA synthesis. In a dietary ALA-supplementation study in dogs (Dunbar et al., 2010), DPA enrichment of the phospholipid and triglyceride fractions, but not the cholesteryl-ester fraction was demonstrated following supplementation, indicating tissue

synthesis and conservation of DPA, which then may serve as a substrate for DHA synthesis in the tissues.

The body preferentially oxidizes FAs with 18 or fewer carbons as an energy source, and uses long-chain PUFAs for building of structural phospholipids, which are components of the cell membrane (Cunnane and Anderson, 1997). Within cell membranes, they have significant effects on membrane integrity and fluidity, as well as cell signaling. In addition, when released from cell membranes by phospholipases, they form eicosanoids through action by the enzymes cyclooxygenase and lipoxygenase. Thus, long-chain PUFAs may exert their effects on target cells by altering the FA composition of membrane phospholipids, thus modifying membrane-mediated processes such as transduction signals, activity of lipases, and synthesis of eicosanoids. Additionally, long-chain PUFAs may regulate gene transcription by direct interaction with specific receptors.

The structural differences between FAs determine their function. For example, both the n3PUFA, EPA, and the n6PUFA, AA, serve as primary precursors for eicosanoids. However, eicosanoids derived from AA are of the 2- and 4-series (e.g. prostaglandin E2, leukotriene B4), whereas eicosanoids derived from EPA are of the 3- and 5-series (e.g. prostaglandin E3, leukotriene B5). Eicosanoids of 3- and 5-series are generally less potent inflammatory mediators compared with the 2- and 4-series (Kang and Weylandt, 2008).

Beneficial effects of n3PUFAs

Immune system

n3PUFA have been reported to have anti-inflammatory effects in multiple clinical studies in humans and in rodent models (Fritsche, 2006). Dietary supplementation with fish oil was reported to improve weight bearing in dogs with naturally-occurring osteoarthritis (Roush et al., 2010).

Several experimental studies illustrate modulation of different aspects of the immune response in healthy dogs by n3PUFA. Dietary fish oil supplementation in dogs was associated with suppressed cell mediated immune-response demonstrated by reduced delayed type hypersensitivity skin response and a low CD4/CD8 T cell ratio (Hall et al., 1999; Hall et al., 2003), decreased lymphocyte proliferation (LeBlanc et al., 2007), reductions in serum Prostaglandin E2 concentrations and IL1 and IL6 activities (LeBlanc et al., 2008), and shifting leukotriene production by neutrophils from the potent chemoattractant Leukotriene B4 towards Leukotriene B5, which is less potent (Hall et al., 2005). The effect of an n3PUFA-rich diet on feline general immune function has been reported in one study to date (Jaso-Friedmann et al., 2008). This study reported comparable lymphocyte proliferation and natural killer cytotoxicity in obese cats fed an n3PUFA- or SFA-rich diet, although there was a trend for higher cytotoxicity in the later.

n3PUFAs also produce anti-inflammatory mediators called resolvins and protectins which may play an important role in the anti-inflammatory actions of these compounds (Kang and Weylandt, 2008).

Lipid metabolism

Prescription grade DHA and EPA are recommended for the treatment of hyperlipidemia in humans (Lombardo and Chicco, 2006; McKenney and Sica, 2007). Similarly, fish oil products

are known to be effective therapy for idiopathic hyperlipidemia in dogs (Schenck, 2006; Xenoulis and Steiner, 2010). However, little is known on the efficacy in cats. Experimental studies on the effect of n3PUFAs supplementation on serum triglyceride and cholesterol concentrations in healthy dogs report variable results. When fish oil (n3PUFA) supplementation was compared to sunflower (n6PUFA) or lard (SFA) supplementation, some studies demonstrated decreases of both triglyceride and cholesterol (Brown et al., 2000b), some showed decrease in cholesterol (Hall et al., 1999) or triglyceride (LeBlanc et al., 2005) only, and some revealed no effect on either cholesterol or triglyceride (Harris, 1997). Studies in healthy cats comparing diets rich in n3PUFAs to diets rich in SFAs (Wilkins et al., 2004; Jordan et al., 2008) or n6PUFAs (Plantinga and Beynen, 2003) revealed no difference in cholesterol or triglycerides.

Possible mechanisms for the hypolipidemic effect of n3PUFAs include suppression of transcription of genes encoding lipogenic enzymes, increased FA oxidation in the liver (Lombardo et al., 1996), stimulation of lipoprotein lipase activity, decrease in intestinal absorption of lipid and glucose (Levy et al., 1993; Thomson et al., 1993), increase in cholesterol secretion into bile (Smit et al., 1991), and suppression of TNF α , which stimulates VLDL secretion from the liver and increases lipolysis in adipose tissue (Feingold and Grunfeld, 1992).

Insulin sensitivity

Epidemiological studies in people show strong association between consumption of fish products and a delay the development of glucose intolerance (Feskens et al., 1991; Lombardo and Chicco, 2006). A positive effect on glucose tolerance has been reported in cats as well; healthy cats fed ad libidum for 21 weeks with n3PUFA-rich diet had lower insulin levels on a glucose tolerance test compared to cats on a SFA-rich diet (Wilkins et al., 2004). In dogs, dietary

supplementation with n3PUFAs did not alter overall insulin sensitivity in 6 healthy non-obese Labrador retriever dogs (Irvine et al., 2002). However, some improvement of insulin sensitivity was noted in a single dog that had a degree of insulin resistance at baseline.

Potential insulin-sensitizing effects of n3PUFAs include up-regulation and activation of PPAR γ by n3PUFA in adipose tissue leading to transformation to small adipocytes that are more sensitive to insulin; increased cell membrane incorporation of n3PUFAs in insulin target tissues resulting in improved insulin activity; n3PUFAs may stimulate FA oxidation in the liver and skeletal muscle, decreasing tissue lipid accumulation and improving insulin action; n3PUFAs may also improve glucose utilization by attenuation of obesity-induced GLUT4 down-regulation in insulin target tissues (Peyron-Caso et al., 2002; Ukropec et al., 2003; Lombardo and Chicco, 2006). The effect of n3PUFAs to improve insulin sensitivity may also be mediated through reduction of adipose tissue TNF α concentrations, as TNF α impairs insulin signaling by inhibition of the tyrosine kinase activity of the insulin receptor (Hotamisligil, 1999).

Cardiovascular system

Epidemiological studies in humans have shown a strong correlation between dietary consumption of fish or other sources of n3PUFAs and reduction in sudden death from myocardial infarction associated with atherosclerosis (von Schacky, 2000). Although naturally-occurring atherosclerosis is uncommon in dogs, experimental studies in dog models of vasoocclusive disease have consistently shown beneficial effects (Oskarsson et al., 1993; Billman et al., 1994; Billman et al., 1999). Dogs with naturally occurring heart failure had lower concentrations of AA, EPA, and DHA than healthy controls. Fish oil supplementation for 8 weeks improved cachexia and was associated with increased survival time (Freeman et al.,

1998). A retrospective study on dogs with heart failure due dilated cardiomyopathy or chronic valvular disease also showed a significantly longer survival time for dogs receiving n3PUFA supplementation (Slupe et al., 2008).

Fish oil has multiple beneficial effects on the cardiovascular system. Heart failure is now known to be an inflammatory disease associated with elevated production of eicosanoids and other inflammatory mediators (von Haehling et al., 2009). Therefore, one of the key beneficial effects of n3PUFA is their anti-inflammatory effect. In dogs with naturally-occurring heart failure, circulating concentrations of TNF α were directly associated with the degree of cardiac cachexia and fish oil supplementation for 8 weeks induced reduction in circulating concentrations of IL1 (Freeman et al., 1998).

Effects of n3PUFAs on cardiac arrhythmias have been demonstrated in humans as well as dogs. Beneficial effects on atrial structural remodeling and atrial fibrillation was demonstrated in a canine model of induced atrial tachypacing (Sakabe et al., 2007). Another study in Boxer dogs with arrhythmogenic right ventricular cardiomyopathy demonstrated decreased number of ventricular premature contractions in 24 hours following supplementation with fish oil compared to sunflower oil or flaxseed oil (Smith et al., 2007). The long-term effects of n3PUFAs on cardiac arrhythmias are consistent with incorporation of FAs into the cell membrane as a mechanism of action. However, some studies in humans and dogs demonstrated acute beneficial effects of n3PUFAs on cardiac arrhythmias, suggesting other mechanisms of action, such as effects on sodium, potassium and calcium channels, are present as well (Billman et al., 1994).

Additional mechanisms that may play a role in the cardioprotective effect of n3PUFAs include reduction of cardiac remodelling and subsequent dysfunction, reduction of heart rate and

blood pressure, improvement of endothelial function, and enhancement of baroreceptor function and heart rate variability (Freeman, 2010).

Renal function

Dietary FA composition has been suggested to modify the course of induced renal disease in rodent models (Barcelli et al., 1986) as well as humans with chronic renal disease (Donadio et al., 1994).

In a retrospective study on cats with naturally-occurring renal failure, the longest median survival time was associated with a diet containing the highest content of EPA (Plantinga et al., 2005). Dogs with experimentally reduced kidney mass receiving supplemental n3PUFA (fish oil) for 20 months had fewer renal structural lesions, less proteinuria, and preservation of glomerular filtration rate when compared with dogs fed a control, low-PUFA-content diet (tallow). In contrast, dogs fed a diet supplemented with n6PUFAs (safflower oil) exhibited progressive deterioration of kidney function associated with proteinuria, morphologic evidence of glomerular and tubulointerstitial injury, and an increased prevalence of end-stage renal failure as compared with dogs fed the same control diet (Brown et al., 1998).

One mechanism for renoprotection by n3PUFA-supplemented diets is their tendency to reduce plasma concentrations of cholesterol and triglycerides. It has been proposed that hypercholesterolemia contributes to progressive kidney injury through deleterious effects of oxidized low-density lipoproteins on endothelial and mesangial cells. Indeed, dogs with experimentally induced renal disease did exhibit a reduction in plasma cholesterol concentration on dietary n3PUFA supplementation (Brown et al., 1998).

Another potential mechanism is the effect of n3PUFAs on glomerular hemodynamics. Dietary supplementation with n6PUFAs in dogs with experimentally reduced renal mass

increased glomerular capillary pressure and the magnitude of glomerular enlargement (Brown et al., 2000b). Supplementation with n3PUFAs leads to a relative decrease in n6PUFA and their effect.

Dietary fish oil supplementation may also provide renoprotection through its ability to suppress inflammation or coagulation by interfering with the production of proinflammatory, procoagulation prostanoids, thromboxanes, and/or leukotrienes. Dogs with early renal insufficiency on dietary fish oil supplementation tended to have a lower urinary prostaglandin E2 and thromboxane A2 excretion and dogs on n6PUFAs supplementation tended to have higher eicosanoid excretion than dogs on SFAs supplementation (Brown et al., 2000b).

Neurologic system

During neural developmental period, DHA is rapidly incorporated into the neural tissues. The high amounts of DHA in the brain and especially in the retina indicate a functional role of this FA in neural tissues. A study investigating retinal function in puppies demonstrated the advantage of dietary DHA (Heinemann et al., 2005). Puppies consuming the highest concentrations of DHA (through fish oil supplementation) in both milk and dry diet consistently demonstrated improved rod sensitivity and elicited the greatest increase in the amplification of the phosphodiesterase cascade compared to puppies consuming a diet low in n3PUFA (tallow). Although visual performance in puppies fed a high-ALA diet (linseed oil) was not significantly lower than in those fed DHA, it was not generally equivalent to the level of retinal function observed in the DHA-fed puppies, suggesting a better effect by DHA over ALA.

1.1.3 Hypotheses

Consumption of dietary n3PUFAs is associated with beneficial health effects including improved insulin sensitivity, decreased tendency for atherosclerosis and heart diseases, and attenuation of hyperlipidemia and inflammatory disorders. Both adiponectin and leptin have insulin-sensitizing and lipid-reducing effects. Adiponectin also has anti-inflammatory and anti-atherosclerotic effects. TNF α and IL6 promote inflammation, insulin resistance and hyperlipidemia. Hence, it may be speculated that the beneficial effects of n3PUFAs are mediated through modulation of these adipokines. Moreover, the finding of inconsistent presence of association between circulating concentrations of adiponectin and body condition in dogs and cats suggests the presence of additional important factors determining adiponectin concentrations. n3PUFAs may potentially serve as one of these factors.

Therefore, our primary hypotheses were:

- Circulating n3PUFAs concentrations are directly related to concentrations of adiponectin and leptin in dogs and cats.
- Dietary supplementation with n3PUFAs in dogs or cats will increase serum concentrations of adiponectin and leptin.
- Treatment of cultured canine and feline adipose tissue with n3PUFA will increase adiponectin and leptin secretion.

In view of the known anti-inflammatory and lipid-reducing effects of n3PUFAs, our additional hypotheses were:

- Circulating n3PUFAs concentrations are inversely related to concentrations of triglyceride and insulin in dogs and cats.
- Circulating n3PUFAs concentrations are inversely related to concentrations of IL6 and TNF α in dogs and cats.
- Treatment of cultured canine and feline adipose tissue with n3PUFA will decrease IL6 and TNF α secretion.

Figure 1. Hypotheses illustration.

A. Adiponectin and leptin have insulin-sensitizing and lipid-lowering effects; adiponectin also has anti-inflammatory and anti-atherosclerotic effects.

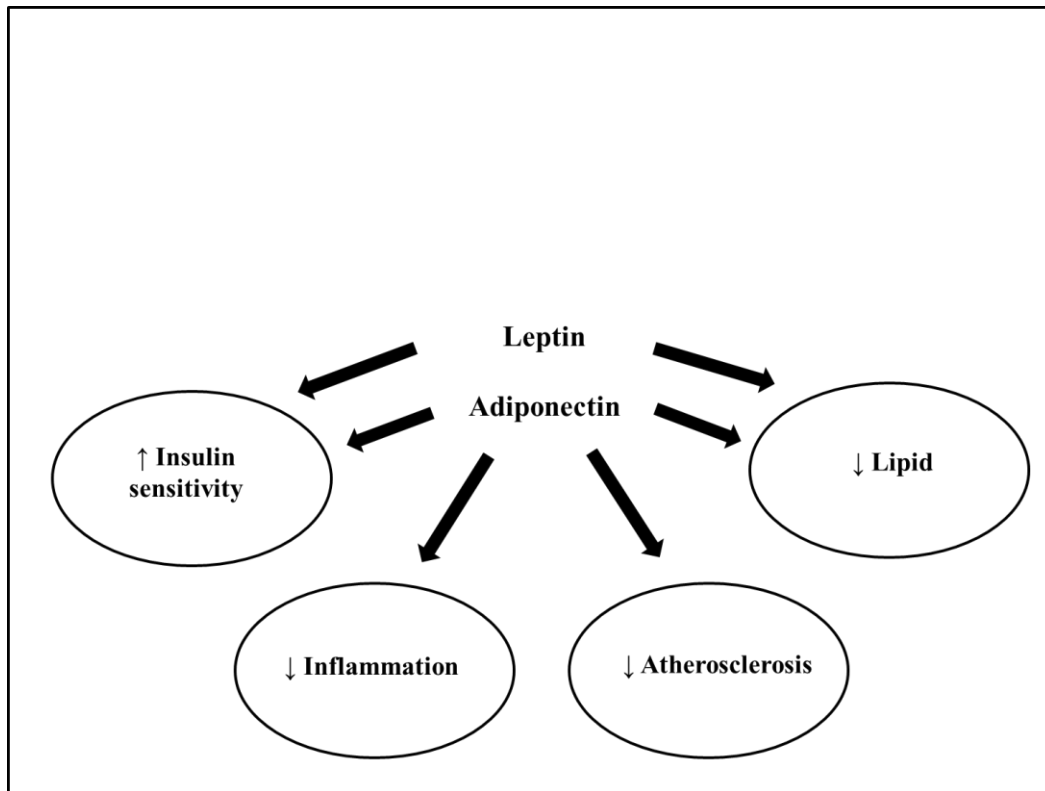
B. IL6 and TNF α promote inflammation, insulin-resistance and hyperlipidemia.

C. n3PUFAs have been shown to have multiple beneficial effects on these metabolic disorders.

D. Therefore, we hypothesized that n3PUFAs have a stimulatory effect on adiponectin and leptin and an inhibitory effect on IL6 and TNF α . Additional aims were to reevaluate the effects of n3PUFAs on concentrations of insulin, triglyceride and cholesterol.

Figure 1 (cont'd)

A.



B.

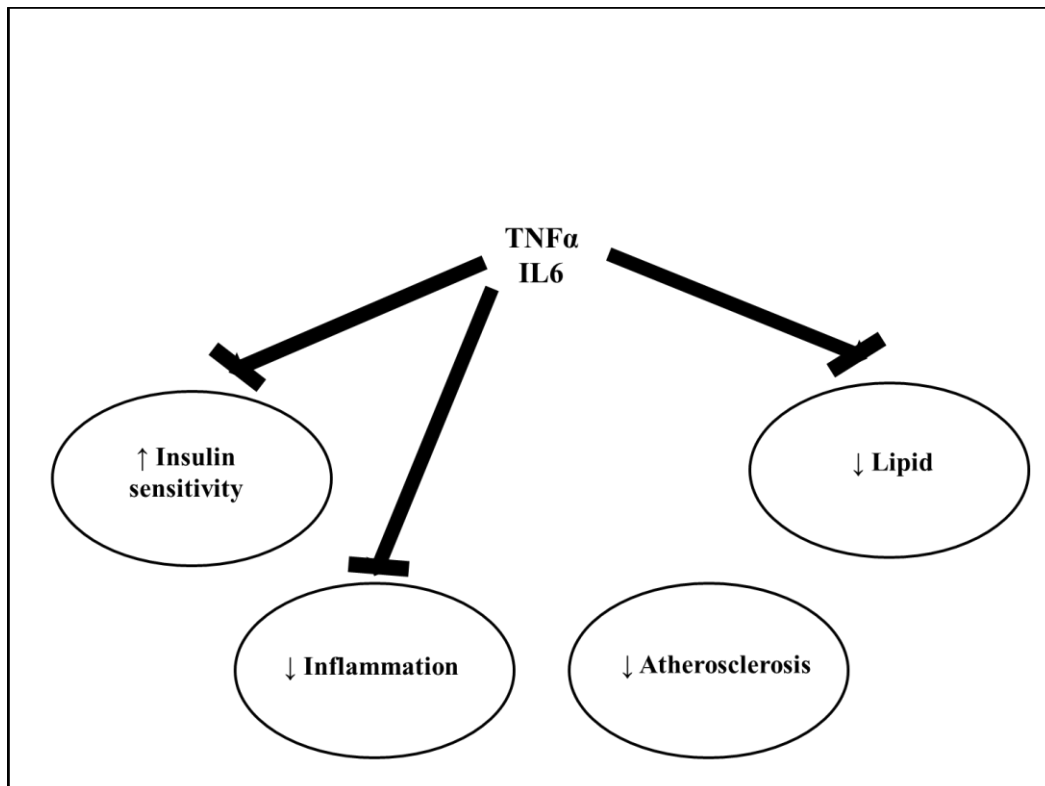
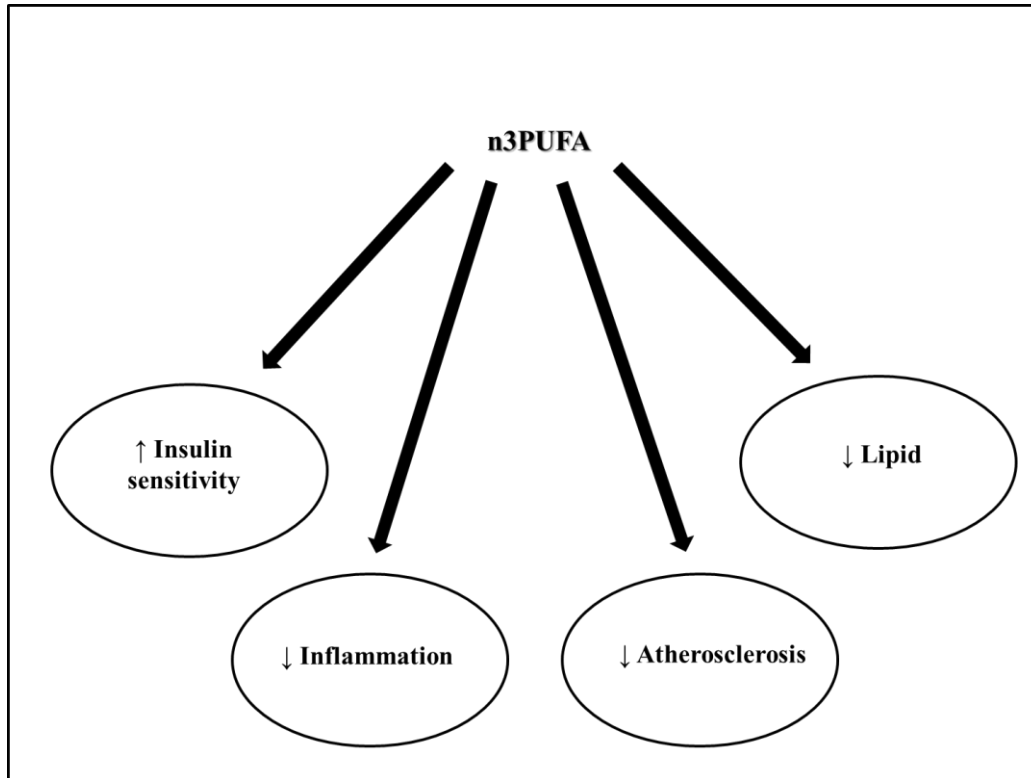
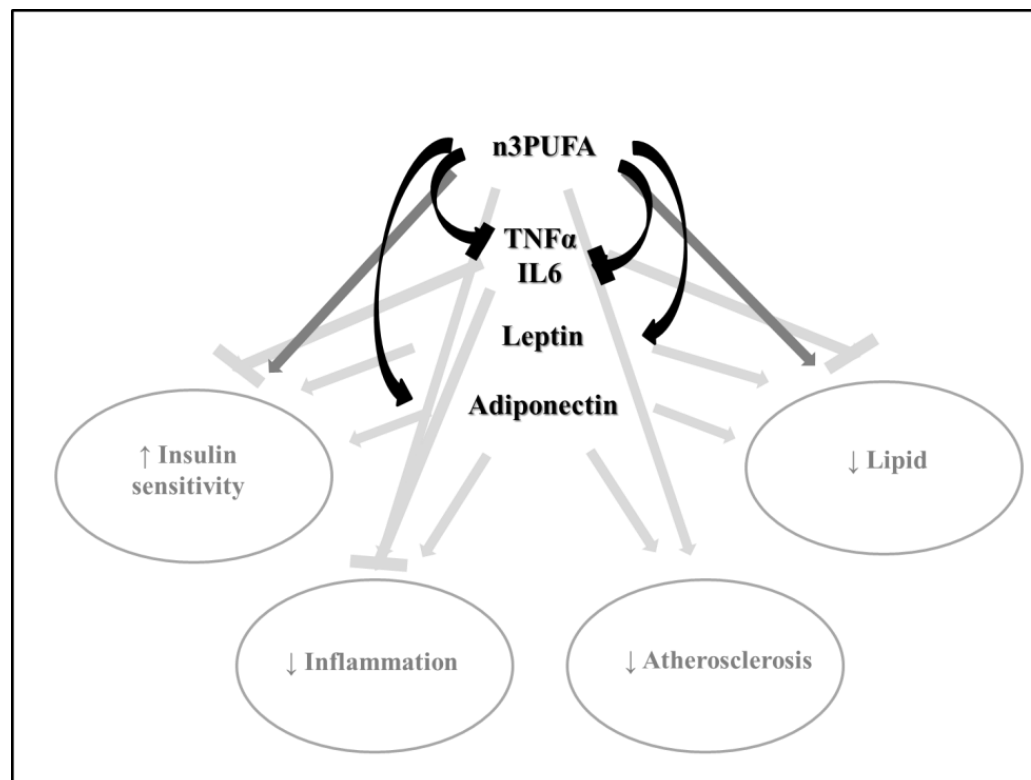


Figure 1 (cont'd)

C.



D.



1.2. Studies: Study aims, methods, and results

1.2.1. Associations among circulating concentrations of adipokines and n3PUFAs, body condition, age, and gender in healthy dogs

Study aims

- Determine associations between circulating concentrations of n3PUFAs and adipokines in healthy dogs.
- Determine associations between circulating concentrations of n3PUFAs and concentrations of insulin, glucose, and triglyceride in healthy dogs.
- Determine associations between potential confounders (body condition, age, and gender) and circulating concentrations of adipokines in healthy dogs.

Methods

Study design

Serum samples were collected following a 12-hour fast from 62 healthy adult (≥ 1 year old) client-owned dogs visiting the Veterinary Teaching Hospital at Michigan State University for routine check-up. The dogs were considered to be healthy based on physical examination and routine biochemistry analysis. Additional inclusion criteria were based on medical and dietary history for a 3 month period preceding sample collection and included: 1) absence of any clinical signs indicating illness; 2) exclusive feeding of a commercially available, nutritionally balanced and complete adult dog diet; and 3) no administration of dietary supplements or medications other than routine anthelmintic drugs. Prior to sample collection, body condition of all dogs was evaluated by a single individual using: 1) Body condition score (BCS) system on a 1- 9 scale (Laflamme, 1997b), and 2) Calculation of an estimated percent body fat (%BF) based on morphological measurements, including pelvic circumference (PC) and hock to stifle distance (HS); % BF-female = $0.93 \times PC - 1.7 \times HS + 5$ or % BF-male = $0.77 \times PC - 1.4 \times HS + 4$ (Burkholder, 1994). The study was performed in compliance with Michigan State University guidelines for research in animals. Informed consent was obtained from all owners.

Serum analysis

Serum was separated immediately following blood collection and frozen at -20°C until analyzed. Serum adiponectin, leptin and insulin concentrations were measured using commercially available assays (Canine Adiponectin ELISA, Millipore; Canine Leptin ELISA, Millipore; Human Insulin RIA, Diagnostic Systems Laboratories- See Appendix C, Protocols 1

and 2). Serum glucose concentrations were measured using an automated chemistry analyzer (AU640^e Chemistry-Immuno System, Olympus America) and concentrations of triglyceride were measured using a spectrophotometric method (Kodak Ektachem DT60 II Clinical Products Division, Eastman Kodak Co). Serum concentrations of IL6 and TNF α were measured in 24 and 17 of the samples, respectively using commercially available assays (Canine IL6 ELISA, R&D Systems; Canine TNF α ELISA, R&D Systems- See Appendix C, Protocol 1). Serum total lipid content was measured and FAs analysis was performed by gas chromatography (See Appendix C, Protocol 3).

Data analysis

Linear correlations among %BF, age, and serum concentrations of adiponectin, leptin, insulin, glucose, and triglyceride were evaluated by use of Pearson's correlation coefficient. Linear correlations between BCS and the other variables were evaluated by use of Spearman correlation coefficient. Natural log transformation was used for the measured analytes and the transformed values as well as %BF and age had normal distribution as determined by P-P plot. Associations between serum concentrations of ALA, EPA, DPA, and DHA and serum concentrations of adiponectin, leptin, insulin, glucose, or triglyceride were evaluated using General linear Model (GLM) including concentrations of each FA, concentration of total lipid, %BF, gender, and age as covariates. Absence of potential multicollinearity among the covariates was confirmed. The additional covariates (%BF, concentration of total lipid, gender, and age) were included to control for potential confounding of the relationship between the FAs and the outcome variables (adiponectin, leptin, insulin, glucose, or triglyceride). All interaction effects between FAs and the additional covariates in the models were assessed. The link function and

the linear predictor for the outcome variables in each of the models are of the form $E[Y|x] = a + bx$. Y is a natural logarithmic transformation of the measured values and has a normal distribution (as confirmed by P–P plots) with a mean ($a + bx$) and constant variances.

Post hoc consideration- Gender did not have a significant effect on serum concentrations of adiponectin, leptin, insulin, triglyceride, and glucose, and therefore was removed from the models. No significant interaction effects between FAs and the additional covariates in the models were present.

Linear correlations between serum concentrations of IL6 or TNF α and other variables were determined using Pearson's correlation. Natural log transformation was used for serum concentrations of insulin, triglyceride, IL6, and TNF α . The transformed values of triglyceride, IL6, and TNF α , as well as the measured values of all other variables had normal distribution as determined by P-P plot. Measured values below the detection limit of the assays were entered as the value of the detection limit of the assay divided by the square root of 2 (Finkelstein and Verma, 2001).

Data were analyzed using a commercially available statistic program (SPSS® 19.0 for Windows). $P < 0.05$ was considered statistically significant.

Results

Dogs

The study population consisted of 18 neutered males, 32 spayed females, 4 intact males, and 8 intact females. Dogs were of various breeds, 12 were mixed breed. The prevalent breeds included Labrador retriever (10), Golden retriever (6), English Cocker Spaniel (5), Australian

shepherd (5), Brittany (3), German Shepherd (3), American Cocker Spaniel (2), Border Collie (2), Rottweiler (2), and Saluki (2).

The dogs' age ranged from 1 to 13 years (median: 5.8 years; interquartile range: 3.9, 8.6), BCS ranged from 4 to 9 (5.5; 5.0, 8.0), and %BF ranged from 2 to 58% (25%, 14, 34). Serum concentrations of total lipid ranged from 5.5 to 17 mg/mL (9.5 mg/dL; 7.8, 11.3). Median (interquartile range) serum concentrations of individual n3PUFAs were 0.00 mg/100 mg FA ALA (0.00 to 0.19), 0.15 mg/100 mg FA EPA (0.00 to 0.49), 1.25 mg/100 mg FA DPA (0.79 to 1.65), 0.8 mg/100 mg FA (0.49 to 1.51) DHA.

Concentrations of glucose ranged from 64 to 113 mg/dL and were above the reference range (70-100 mg/dL) in 10 of the dogs and below it in 1 dog. Concentrations of triglyceride ranged from 22 to 214 mg/dL and were above the reference range (21-116 mg/dL) in 10 of the dogs.

Linear correlations among measures of body condition, age, and circulating concentrations of adipokines, insulin, glucose, and triglyceride

Correlation coefficients for the analysis of all dogs are presented in Table 1. Correlation coefficients for separate analyses of non-obese (BCS = 4-6) and obese (BCS = 7-9) dogs are presented in Table 2.

Table 1. Correlation coefficients among measures of body condition, age, and circulating concentrations of adipokines, insulin, glucose, and triglyceride in 62 healthy dogs.

Table 1 (cont'd)

%BF	<i>r</i> <i>P</i>	0.81 <0.001						
Age	<i>r</i> <i>P</i>	0.33 0.008	0.36 0.004					
Adp	<i>r</i> <i>P</i>	-0.38 0.003	-0.46 <0.001	-0.48 <0.001				
Lep	<i>r</i> <i>P</i>	0.79 <0.001	0.78 <0.001	0.45 <0.001	-0.41 0.001			
Ins	<i>r</i> <i>P</i>	0.51 <0.001	0.61 <0.001	0.41 0.001	-0.50 <0.001	0.57 <0.001		
TG	<i>r</i> <i>P</i>	0.57 <0.001	0.75 <0.001	0.45 <0.001	-0.47 <0.001	0.68 <0.001	0.64 <0.001	
Glu	<i>r</i> <i>P</i>	0.06 0.7	0.22 0.089	-0.16 0.2	-0.13 0.3	0.22 0.097	0.27 0.039	0.23 0.082
		BCS	%BF	Age	Adp	Lep	Ins	TG

Adp- Adiponectin; Lep- Leptin; Ins- Insulin; TG- triglyceride; Glu- Glucose

Table 2. Correlation coefficients among measures of body condition, age, and circulating concentrations of adipokines, insulin, glucose, and triglyceride in 24 obese and 38 non-obese healthy dogs.

Table 2 (cont'd)

		BCS	%BF	Age	Adp	Lep	Ins	TG	Glu	
BCS	<i>r</i> <i>P</i>		0.63 0.001	-0.16 0.4	-0.07 0.8	0.65 0.001	0.22 0.3	0.23 0.3	-0.01 1.0	Obese (n=24)
%BF	<i>r</i> <i>P</i>	0.37 0.02		-0.31 0.1	0.16 0.5	0.54 0.006	0.46 0.025	0.48 0.017	0.10 0.6	
Age	<i>r</i> <i>P</i>	0.12 0.48	0.42 0.009		-0.28 0.2	-0.07 0.7	0.16 0.5	-0.29 0.2	-0.27 0.2	
Adp	<i>r</i> <i>P</i>	-0.14 0.4	-0.51 0.001	-0.44 0.005		-0.09 0.7	-0.39 0.058	0.07 0.8	0.13 0.5	
Lep	<i>r</i> <i>P</i>	0.33 0.053	0.59 <0.001	0.45 0.006	-0.29 0.084		0.34 0.1	0.40 0.053	0.15 0.5	
Ins	<i>r</i> <i>P</i>	0.26 0.1	0.40 0.014	0.40 0.014	-0.41 0.012	0.44 0.007		0.49 0.015	-0.004 1.0	
TG	<i>r</i> <i>P</i>	0.03 0.9	0.62 <0.001	0.58 <0.001	-0.50 0.002	0.49 0.002	0.52 0.001		0.14 0.5	
Glu	<i>r</i> <i>P</i>	-0.15 0.4	0.16 0.4	-0.25 0.1	-0.13 0.4	0.13 0.5	0.37 0.026	0.16 0.4		
Non-obese (n=38)										

Adp- Adiponectin; Lep- Leptin; Ins- Insulin; TG- triglyceride; Glu- Glucose

n3PUFAs and total lipid

Serum concentrations of DPA were significantly positively related to concentrations of adiponectin and leptin and negatively related to concentrations of triglyceride following adjustment for %BF, age, total lipid, and the other n3PUFAs (ALA, EPA, DHA) (Table 3). An increase of 1 mg/100 mg FA in serum concentration of DPA was associated with 68% increase in the adjusted geometric mean of concentrations of adiponectin, 51% increase in the adjusted geometric mean of concentrations of leptin, and 22% decrease in the adjusted geometric mean of

concentrations of triglyceride. There were no significant associations between serum concentrations of DPA and insulin or glucose following adjustment for potential confounders (Table 3).

Table 3. Associations between DPA and adiponectin, leptin, insulin, glucose, and triglyceride in 62 healthy dogs.*

Variable	Mean (95% CI) [†]	Fold increase (95% CI) [‡]	<i>P</i>	Partial η^2
Adiponectin	7.8 µg/mL (6.9, 8.8)	1.68 (1.27, 2.22)	<0.001	0.21
Leptin	5.8 ng/mL (4.9, 7.0)	1.51 (1.04, 2.21)	0.032	0.08
Insulin	124 pmol/L (110, 139)	0.99 (0.76, 1.29)	0.9	0.00
Glucose	87 mg/dL (84, 91)	0.98 (0.90, 1.07)	>0.1	0.00
Triglyceride	65 mg/dL (60, 70)	0.78 (0.65, 0.92)	0.005	0.14

* Covariates in the GLM include: %BF, age, total lipid, ALA, DPA, EPA, and DHA

[†] Covariates in the model are evaluated at their mean values as follows; age: 6 years, %BF: 25%, total lipid: 9.7 mg/mL, ALA: 0.14 mg/100 mg FA, EPA: 0.33 mg/100 mg FA, DPA: 1.29 mg/100 mg FA, and DHA: 1.06 mg/100 mg FA.

[‡] Fold increases and 95% CI in adjusted geometric means of serum concentration of each variable with an increase of 1 mg/100 mg FA in serum concentration of DPA.

Serum concentrations of ALA were significantly associated to concentrations of triglyceride following adjustment %BF, age, total lipid, and the other n3PUFAs (EPA, DPA, DHA). An increase of 1 mg/100 mg FA in concentrations of ALA was associated with a 56% increase in the adjusted geometric mean of serum concentration of triglyceride (95% CI of fold

increase of adjusted geometric mean: 1.15, 2.10; $P = 0.005$; Partial $\eta^2 = 0.14$). There were no significant associations between serum concentrations of ALA and adiponectin, leptin, insulin, or glucose. No significant associations between serum concentrations of EPA or DHA and concentrations of adiponectin, leptin, insulin, triglyceride or glucose were present.

The associations between total lipid and insulin and triglyceride remained significant following adjustment for %BF, age, and n3PUFAs. An increase of 1 mg/mL in serum concentrations of total lipid was associated with 7% increase in the adjusted geometric mean of concentrations of insulin, and 6% increase in the adjusted geometric mean of concentrations of triglyceride. No significant association between total lipid and adiponectin, leptin, or glucose was present following adjustment for potential confounders (Table 4).

Table 4. Associations between total lipid and adiponectin, leptin, insulin, glucose, and triglyceride in 62 healthy dogs.*

Variable	Fold increase (95% CI)†	<i>P</i>	Partial η^2
Adiponectin	0.97 (0.92, 1.03)	0.3	0.02
Leptin	1.02 (0.95, 1.11)	0.6	0.01
Insulin	1.07 (1.01, 1.12)	0.014	0.11
Glucose	1.01 (0.99, 1.03)	0.5	0.01
Triglyceride	1.06 (1.03, 1.10)	0.001	0.19

* Covariates in the GLM include: %BF, age, total lipid, ALA, EPA, DPA, and DHA.

† Fold increases and 95% CI in adjusted geometric means of serum concentration of each variable with an increase of 1 mg/mL in serum concentration total lipid.

Body condition and age

The associations between %BF and leptin, insulin, and triglyceride remained significant following adjustment for age, total lipid, and n3PUFAs. An increase of 1%BF was associated with 7% increase in the adjusted geometric mean of concentrations of leptin, 2% increase in the adjusted geometric mean of concentrations of insulin, and 2% increase in the adjusted geometric mean of concentrations of triglyceride. No significant associations between %BF and adiponectin or glucose was present following adjustment for potential confounders (Table 5).

Table 5. Associations between %BF and adiponectin, leptin, insulin, glucose, and triglyceride in 62 healthy dogs.*

Variable	Fold increase (95% CI)†	<i>P</i>	Partial η^2
Adiponectin	0.99 (0.98, 1.01)	0.4	0.01
Leptin	1.07 (1.05, 1.08)	<0.001	0.50
Insulin	1.02 (1.01, 1.04)	<0.001	0.22
Glucose	1.00 (1.00, 1.01)	0.093	0.05
Triglyceride	1.02 (1.01, 1.03)	<0.001	0.32

* Covariates in the GLM include: %BF, age, total lipid, ALA, EPA, DPA, and DHA.

† Fold increases and 95% CI in adjusted geometric means of serum concentrations of each variable with increase of 1% BF.

The associations between age and adiponectin, leptin, insulin, and triglyceride remained significant following adjustment for %BF, total lipid, and n3PUFAs. An increase of 1 year of age was associated with 8% decrease in the adjusted geometric mean of concentrations of adiponectin, 8% increase in the adjusted geometric mean of concentrations of leptin, 4% increase in the adjusted geometric mean of concentrations of insulin, and 4% increase in the adjusted geometric mean of concentrations of triglyceride. No significant association between age and glucose was present following adjustment (Table 6).

Table 6. Associations between age and adiponectin, leptin, insulin, glucose, and triglyceride in 62 healthy dogs.*

Variable	Fold increase (95% CI)†	<i>P</i>	Partial η^2
Adiponectin	0.92 (0.88, 0.96)	0.001	0.20
Leptin	1.08 (1.01, 1.16)	0.018	0.10
Insulin	1.04 (1.00, 1.09)	0.045	0.07
Glucose	0.99 (0.97, 1.00)	0.083	0.06
Triglyceride	1.04 (1.01, 1.07)	0.014	0.11

* Covariates in the GLM include: %BF, age, total lipid, ALA, EPA, DPA, and DHA.

† Fold increases and 95% CI in adjusted geometric means of serum concentrations of each variable with increase of 1 year of age.

Associations with serum concentrations of IL6 and TNF α

Fourteen of the 24 and 2 of the 17 measured values of IL6 and TNF α , respectively, were below the assays detection limits. Concentrations of IL6 and TNF α were significantly correlated ($r = 0.70$, $P = 0.002$). Concentrations of IL6 were significantly correlated to %BF ($r = -0.42$, $P = 0.041$), DPA ($r = -0.41$, $P = 0.046$), n6/n3 ratio ($r = 0.41$, $P = 0.047$), and serum concentrations of insulin ($r = 0.46$, $P = 0.031$). Concentrations of TNF α were significantly correlated to PA ($r = 0.49$, $P = 0.045$).

1.2.2. Effect of fish oil supplementation on concentration of adipokines in healthy dogs

Study aim

- Determine the effect of dietary supplementation with fish oil on circulating concentrations of adipokines in healthy dogs.
- Determine the effect of dietary supplementation with fish oil on circulating concentrations of triglyceride, cholesterol, insulin, and glucose in healthy dogs.

Methods

Study design

Twenty healthy adult (≥ 1 year old) dogs (a subgroup of the dogs enrolled in Study 1.2.1) were enrolled in the study. The dogs were considered to be healthy based on physical examination and routine serum biochemistry analysis. Additional inclusion criteria were based on medical and dietary history for a 3 month period preceding sample collection and included: 1) absence of any clinical signs indicating illness; 2) exclusive feeding of a commercially available, nutritionally balanced and complete adult dog diet; and 3) no administration of dietary supplements or medications other than routine anthelmintic drugs. All dogs were administered a fish oil supplement (Enteric Coated Omega 3 Fish Oil softgel, Spring Valley) containing 120 mg DHA, 180 mg EPA and 500 mg SFAs in 1g fat at a dose of 220 mg/kg (66 mg/kg DHA and EPA) daily for 30 days.

At baseline (T0) and after 30 days of fish oil supplementation (T1), fasted serum samples were collected, body weight was recorded, and body condition was evaluated in all dogs. Evaluation of body condition was performed using a BCS system on a 1–9 scale (Laflamme, 1997b) and a calculated estimate of %BF based on morphometric measurements, including pelvic circumference (PC) and hock to stifle distance (HS) as follows: %BF(female) = $0.93 \times PC - 1.7 \times HS + 5$ or % BF(male) = $0.77 \times PC - 1.4 \times HS + 4$ (Burkholder, 1994). Additional fasted blood samples collection and evaluation of body weight and body condition were performed in 17 of the dogs at least 10 weeks after discontinuation of the fish oil supplement (T2). Dogs continued to consume their pre-study diet and no dietary changes were made throughout the

study duration. The study was performed in compliance with Michigan State University guidelines for research in animals. Written informed consent was obtained from all owners.

Serum analysis

Blood samples collected at T0 and T1 were analyzed for concentrations of adiponectin, leptin, insulin, glucose, cholesterol, triglyceride, and FAs. Concentrations of adiponectin were additionally measured in blood samples collected at T2.

Serum was separated immediately following blood collection and frozen at -20 °C until analyzed. Serum adiponectin, leptin and insulin concentrations were measured using commercially available assays (Canine Adiponectin ELISA, Millipore; Canine Leptin ELISA, Millipore; Human Insulin RIA, Diagnostic Systems Laboratories- See Appendix C, Protocols 1 and 2). Serum concentrations of glucose, cholesterol, and triglyceride were measured using a spectrophotometric method (Kodak Ektachem DT60 II Clinical Products Division, Eastman Kodak Co). Serum total lipid content was measured and FAs analysis was performed by gas chromatography (See Appendix C, Protocol 3).

Data analysis

T0 values of calculated %BF and serum concentrations of adiponectin, leptin, insulin, glucose, triglyceride, cholesterol, and FAs were compared to T1 values or T2 values using paired *t*-test. Additionally, these variables and the absolute difference in adiponectin concentrations from T0 to T1 were compared between 2 subgroups of dogs with different pattern of response to the supplement using independent sample *t*-test. Linear correlations were determined using Pearson's correlation. Natural log transformation was used for triglyceride. The transformed

triglyceride values as well as the measured values of all other variables had normal distribution as determined by Shapiro-Wilk test.

Data were analyzed using a commercially available statistic program (SPSS 19.0 for Windows). $P < 0.05$ was considered significant. Results are reported as mean \pm standard deviation.

Results

Dogs

The study dogs included 10 females (3 intact) and 10 males (3 intact). Age ranged from 1 to 12 years (Median: 4 years) and BCS ranged from 4 to 6 (Median: 5). Breeds included Labrador retriever (6), Australian shepherd (4), Border Collie (2), English Cocker Spaniel (2), Saluki (2), and 1 of each of the following: Siberian Husky, German shepherd, Vizsla, and mixed breed. One dog was reported to have mild flatulence during the fish oil supplementation period. No other adverse effects were noted.

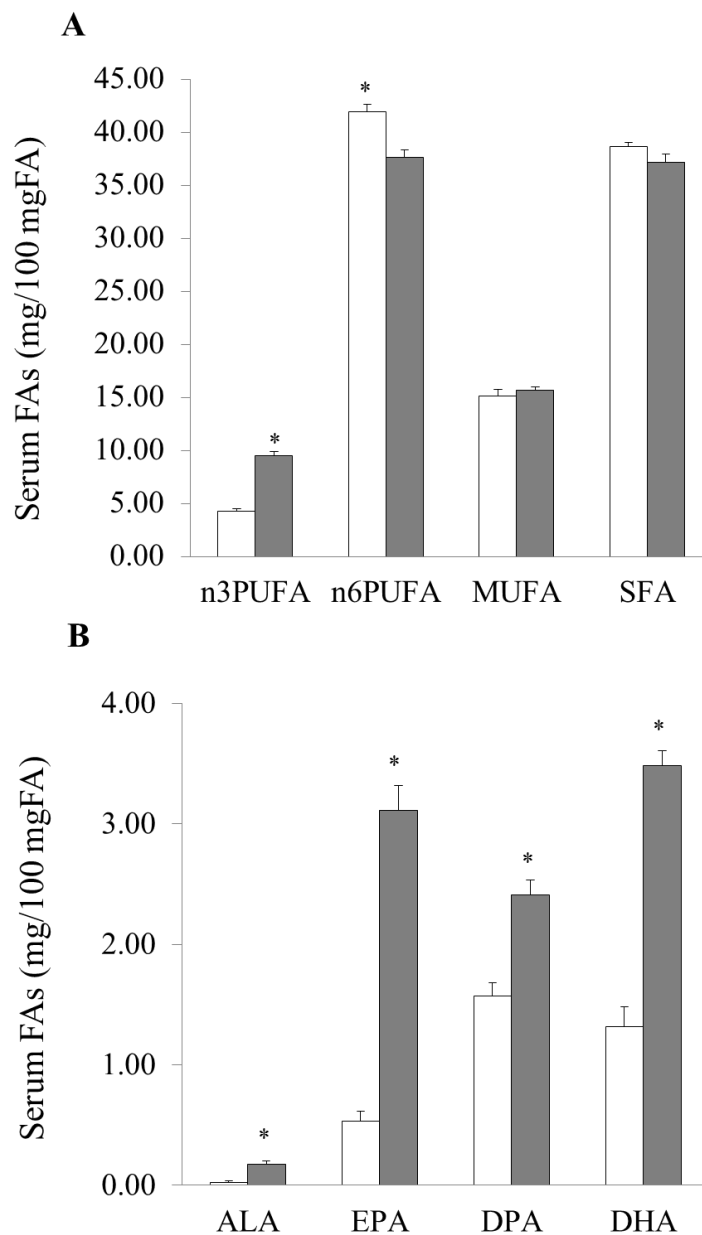
Effect of supplementation

Mean body weight and mean %BF did not change significantly between T0 and T1 (23.1kg \pm 5.9 vs. 23.1kg \pm 5.8; $P = 1.0$ and 15.2% \pm 7.4 vs. 16.1% \pm 8.6; $P = 0.5$, respectively) or between T0 and T2 (24.3kg \pm 5.4 vs. 24.5kg \pm 5.7; $P = 0.6$ and 15.4% \pm 7.9 vs. 15.8% \pm 7.5; $P = 0.8$, respectively). Serum concentrations of total n3PUFA as well as concentrations of individual n3PUFAs increased significantly following supplementation (Figure 2A and 2B).

Concentrations of total n6PUFA decreased significantly following supplementation, while there were no significant changes in concentrations of total MUFAs and total SFAs (Figure 2A).

Figure 2. Serum concentration of total FAs within categories (A) and individual n3PUFAs (B) at baseline (□) and 30 days after fish oil supplementation (■) in 20 healthy dogs.†

† Bar represents the mean and error bar represents 1 standard error; * $P < 0.001$ compared to baseline.

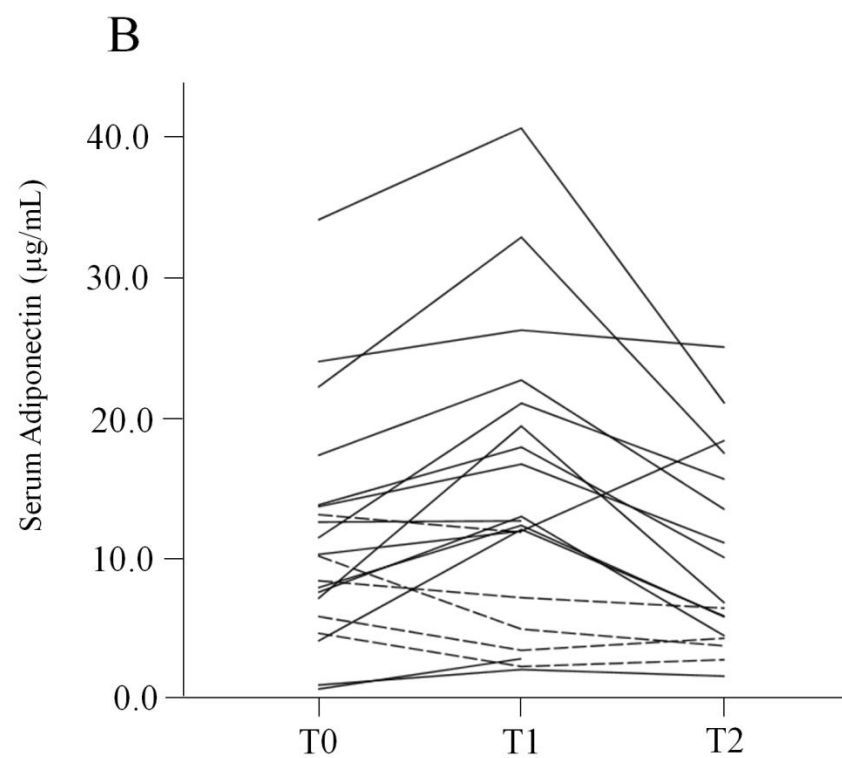
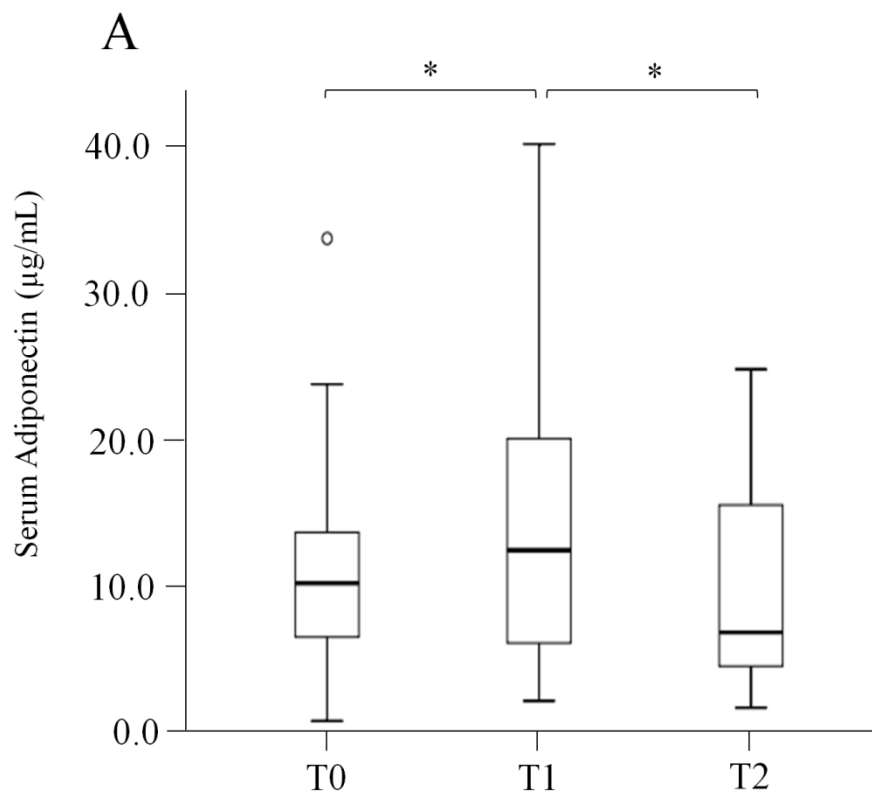


Mean adiponectin concentration at T1 ($14.7 \mu\text{g/mL} \pm 10.3$) was significantly ($P = 0.006$) higher than T0 ($11.5 \mu\text{g/mL} \pm 8.1$). Mean adiponectin concentration at T2 ($10.3 \mu\text{g/mL} \pm 7.0$) was not significantly different ($P = 0.1$) from T0 ($12.0 \mu\text{g/mL} \pm 8.4$) (Fig 3A). Post supplementation, concentration of adiponectin increased in 15 dogs and decreased in 5 dogs (Figure 3B). The mean change in dogs that had increased adiponectin concentrations at T1 was significantly larger than the mean change in dogs that had decreased concentrations at T1 ($5.1 \mu\text{g/mL} \pm 3.7$ vs. $2.5 \mu\text{g/mL} \pm 1.6$; $P = 0.044$). Mean baseline %BF was significantly ($P = 0.032$) higher in dogs that had decreased adiponectin concentration at T1 ($21.2\% \pm 7.5$) as compared to dogs that had increased adiponectin concentration at T1 ($13.2\% \pm 6.4$). No significant differences in mean baseline concentrations of adiponectin ($P = 0.3$), leptin ($P = 0.7$), insulin ($P = 0.2$), glucose ($P = 0.3$), triglyceride ($P = 0.6$), cholesterol ($P = 0.2$), and total n3PUFAs ($P = 0.8$) were present between the dogs with increased and the dogs with decreased concentrations of adiponectin at T1.

Figure 3. Serum concentrations of adiponectin in 20 healthy dogs at baseline (T0), 30 days after fish oil supplementation (T1), and following discontinuation of supplementation (T2).†

† A) The horizontal bar within the box plot indicates the median, the box contains the middle half of the results, the T-bars indicate the range, and the circle indicates outlying data point. * $P < 0.01$. B) The lines outline changes in individual dogs, where continuous and broken lines respectively represent increases and decreases in concentration from T0 to T1.

Figure 3 (cont'd)



No significant differences from T0 to T1 were present in mean concentrations of leptin (4.0 ng/mL \pm 2.7 vs. 4.6 ng/mL \pm 2.9; $P = 0.2$), insulin (94 pmol/L \pm 40 vs. 90 pmol/L \pm 43; $P = 0.8$), glucose (85 mg/dL \pm 8 vs. 90 mg/dL \pm 13; $P = 0.2$), ln-triglyceride (3.66 \pm 0.3 vs. 3.73 \pm 0.34; $P = 0.4$), and cholesterol (221 mg/dL \pm 64 vs. 216 mg/dL \pm 52; $P = 0.7$).

Linear correlations among variables at baseline and post supplementation

Concentrations of adiponectin were significantly correlated to concentrations of total n3PUFAs and DPA at T0 ($r = 0.47$, $P = 0.037$ and $r = 0.52$, $P = 0.019$, respectively) and T1 ($r = 0.47$, $P = 0.036$ and $r = 0.49$, $P = 0.029$, respectively), and were nearly significant to %BF at T0 ($r = -0.43$, $P = 0.06$) but not at T1 ($r = -0.2$, $P = 0.4$). %BF was significantly correlated to concentrations of leptin and triglyceride at T0 ($r = 0.48$, $P = 0.038$ and $r = 0.66$, $P = 0.003$, respectively) and at T1 ($r = 0.6$, $P = 0.008$ and $r = 0.7$, $P = 0.001$, respectively).

1.2.3. Associations among circulating concentrations of adipokines and n3PUFAs, body condition, age, and gender in healthy cats

Study aims

- Determine associations between circulating concentrations of n3PUFAs and adipokines in healthy cats.
- Determine associations between circulating concentrations of n3PUFAs and concentrations of insulin, glucose, and triglyceride in healthy cats.
- Determine associations between potential confounders (BCS, age and gender) and circulating concentrations of adipokines in healthy cats.

Methods

Study design

Serum samples were collected following a 12-hour fast from 56 healthy adult (≥ 1 year old) client-owned cats visiting the Veterinary Teaching Hospital at Michigan State University for routine check-up. The cats were considered to be healthy based on physical examination and routine biochemistry analysis. Additional inclusion criteria were based on medical and dietary history for a 3 month period preceding sample collection and included: 1) absence of any clinical signs indicating illness; 2) exclusive feeding of a commercially available, nutritionally balanced and complete adult cat diet; 3) no administration of dietary supplements or medications. Mild hyperglycemia (up to 9.0 mmol/L) was not an exclusion criterion. This magnitude of increase in glucose concentration was considered to represent stress hyperglycemia consistent with a reported mean peak glucose concentration of 9.0 mmol/L in healthy cats with normal glucose tolerance test subjected to an acute stressor (Rand et al., 2002). Prior to sample collection, body condition of all cats was evaluated by a single individual using: 1) BCS system on a scale of 1 to 9 (Laflamme, 1997a), and 2) Calculation of an estimated %BF based on morphological measurements, including thoracic circumference (TC) and hock to stifle distance (HS); % BF = $(1.54 \times \text{TC}) - (1.58 \times \text{HS}) - 8.67$ (Hawthorne and Butterwick, 2000). The study was performed in compliance with Michigan State University guidelines for research in animals. Informed consent was obtained from all owners.

Serum analysis

Serum was separated immediately following blood collection and frozen at -20 °C until analyzed. Serum adiponectin, leptin and insulin concentrations were measured using commercially available assays (Mouse/rat Adiponectin ELISA kit, B-Bridge, Multispecies Leptin RIA kit, Millipore, and Human Insulin RIA kit, Diagnostic Systems Laboratories- See Appendix C, Protocols 1 and 2) previously used in cats and validated in our laboratory (Backus et al., 2000; Hoenig et al., 2007). Serum glucose concentrations were measured using an automated chemistry analyzer (AU640^e Chemistry-Immuno System, Olympus), and concentrations of cholesterol and triglyceride were measured using a spectrophotometric method (Kodak Ektachem DT60 II Clinical Products Division, Eastman Kodak Co). Serum FA analysis was performed by gas chromatography (See Appendix C, Protocol 3).

Data analysis

Linear correlations among %BF, age, and serum concentrations of adiponectin, leptin, insulin, glucose, and triglyceride were evaluated by use of Pearson's correlation coefficient. Linear correlations between BCS and the other variables were evaluated by use of Spearman correlation coefficient. Natural log transformation was used for the measured analytes and the transformed values as well as %BF and age had normal distribution as determined by P-P plot. Associations between serum concentrations of DHA, DPA, EPA, and ALA and serum concentrations of adiponectin, leptin, insulin, glucose, triglyceride, or cholesterol were evaluated using GLM including DHA, DPA, EPA, ALA, BCS, gender, and age as covariates. Absence of potential colinearity among serum concentrations of these individual FA was confirmed. The additional covariates (BCS, gender, and age) were included to control for potential confounding of the relationship between EPA, DPA, DHA, or ALA and the outcome variables (adiponectin,

leptin, insulin, glucose, triglyceride, or cholesterol). The link function and the linear predictor for each of the variables are of the form $E[Y|x] = a + bx$. Y is a natural logarithmic transformation of the measured values and has a normal distribution (as confirmed by P-P plots) with a mean ($a + bx$) and constant variances. All interaction effects between individual FA and other variables in the models were assessed.

Post hoc categorization - Significant or nearly significant interaction effects were present between BCS and ALA in the analysis of the association with TG ($P=0.048$), between BCS and EPA in the analyses of the associations with adiponectin ($P=0.052$), insulin ($P=0.038$), and triglyceride ($P<0.001$), between BCS and DPA in the analysis of the associations with triglyceride ($P=0.001$), and between BCS and DHA in the analyses of the associations with adiponectin ($P=0.067$) and triglyceride ($P=0.018$). Therefore analyses were performed separately for non-obese (BCS 4-6) and obese (BCS 7-8) cats. Categorization into these 2 groups was used due to insufficient statistical power of the study to evaluate the effect of individual n3PUFA on the outcome variables in separate analyses of each BCS level with age and gender as additional covariates, and was based on the tendency for a positive or negative association between EPA and adiponectin in these separate analyses: exponentiated coefficients for the association between EPA and adiponectin in the analyses for BCS 4, 5, 6, 7, and 8 were 0.11 ($P>0.1$), 0.42 ($P<0.1$), 0.87 ($P>0.1$), 5.39 ($P<0.1$), and 1.91 ($P>0.1$), respectively. ALA and DPA were not included in the separate models due to insignificant effect on any of the outcome variables and since their inclusion decreased the models adjusted R^2 .

Data were analyzed using a commercially available statistic program (SPSS 19.0 for Windows). $P < 0.05$ was considered statistically significant.

Results

Cats

One neutered female cat was excluded from the study due to the presence of fasting hyperglycemia (serum glucose concentration: 14.8 mmol/L). BCSs of the 55 cats included in the study were 4 (5 cats), 5 (15 cats), 6 (14 cats), 7 (9 cats), and 8 (12 cats). Hence, 34 cats were categorized as non-obese and 21 as obese. Fourteen of the non-obese cats were neutered males and 20 were neutered females. Sixteen of the obese cats were neutered males and 5 were neutered females. The non-obese group included 26 domestic short hair, 3 domestic long hair cats, 2 mixed breed cats, 1 Siamese, 1 Birman, and 1 Bengal cat. The obese group included 14 domestic short hair, 4 domestic long hair, 2 domestic medium hair, and 1 mixed breed cat.

Age of the non-obese cats ranged from 1 to 16 years (Median: 3.0; interquartile range: 1.5-6.5) and that of the obese cats from 1 to 9 years (5.0 years; 3.5-7.0). Median (interquartile range) of serum concentrations of ALA, EPA, DPA, and DHA were 0.21 mg/100mg FA (0.00-0.35), 0.34 mg/100mg FA (0.00-0.48), 0.53 mg/100mg FA (0.12-0.57), and 1.75 mg/100mg FA (1.37- 2.82), respectively, in the non-obese group, and 0.36 mg/100mg FA (0.28-0.56), 0.21 mg/100mg FA (0.06-0.44), 0.47 mg/100mg FA (0.36-0.68), and 2.15 mg/100mg FA (1.29-2.66), respectively, in the obese group.

Serum glucose concentrations were above the reference range (3.3-7.5 mmol/L) in 5 non-obese and 2 obese cats. Serum triglyceride concentrations were above the reference range (21-155 mg/dL) in 5 cats in the obese group and were within the range in all cats in the non-obese group. Serum concentrations of cholesterol were within the reference range (77-306 mg/dL) in all cats.

Linear correlations among measures of body condition, age, and circulating concentrations of adipokines, insulin, glucose, and triglyceride

Correlation coefficients for the analysis of all cats are presented in Table 7. Correlation coefficients for separate analyses of non-obese (BCS=4-6) and obese (BCS=7-8) cats are presented in Table 8.

Table 7. Correlation coefficients among measures of body condition, age, and circulating concentrations of adipokines, insulin, glucose, and triglyceride in 55 healthy cats.

%BF	<i>r</i> <i>P</i>	0.82 <0.001						
Age	<i>r</i> <i>P</i>	-0.13 0.4	-0.03 0.9					
Adp	<i>r</i> <i>P</i>	-0.50 <0.001	-0.55 <0.001	-0.03 0.8				
Lep	<i>r</i> <i>P</i>	0.46 <0.001	0.57 <0.001	0.2 0.1	-0.37 0.005			
Ins	<i>r</i> <i>P</i>	0.50 <0.001	0.51 <0.001	-0.1 0.3	-0.36 0.007	0.42 0.001		
TG	<i>r</i> <i>P</i>	0.46 <0.001	0.43 0.001	-0.2 1.0	-0.27 0.044	0.24 0.081	0.43 0.001	
Glu	<i>r</i> <i>P</i>	-0.15 0.3	-0.15 0.3	-0.19 0.2	-0.01 1.0	-0.22 1.0	-0.01 1.0	0.20 0.1
		BCS	%BF	Age	Adp	Lep	Ins	TG

Adp- Adiponectin; Lep- Leptin; Ins- Insulin; TG- triglyceride; Glu- Glucose

Table 8. Correlation coefficients among measures of body condition, age, and circulating concentrations of adipokines, insulin, glucose, and triglyceride in 21 obese and 34 non-obese healthy cats.

		BCS	%BF	Age	Adp	Lep	Ins	TG	Glu	
BCS	<i>r</i> <i>P</i>		0.73 <0.001	0.18 0.4	-0.80 <0.001	0.70 <0.001	0.52 0.015	0.25 0.3	-0.03 0.9	Obese (n=21)
%BF	<i>r</i> <i>P</i>	0.67 <0.001		0.25 0.3	-0.69 0.001	0.61 0.004	0.44 0.045	0.18 0.4	-0.10 0.7	
Age	<i>r</i> <i>P</i>	-0.28 0.1	-0.20 0.3		-0.20 0.4	0.18 0.4	0.27 0.2	-0.15 0.5	-0.01 1.0	
Adp	<i>r</i> <i>P</i>	-0.13 0.5	-0.11 0.5	0.05 0.8		-0.52 0.016	-0.53 0.014	-0.29 0.2	-0.28 0.2	
Lep	<i>r</i> <i>P</i>	0.04 0.8	0.31 0.074	0.31 0.075	-0.048 0.8		0.64 0.002	0.13 0.6	-0.38 0.093	
Ins	<i>r</i> <i>P</i>	0.41 0.017	0.32 0.062	-0.37 0.03	-0.04 0.8	0.10 0.6		0.38 0.087	0.27 0.2	
TG	<i>r</i> <i>P</i>	0.20 0.3	0.15 0.4	-0.36 0.039	-0.16 0.4	0.00 1.0	0.27 0.1		0.28 0.2	
Glu	<i>r</i> <i>P</i>	0.26 0.1	-0.14 0.4	-0.29 0.092	-0.11 0.6	-0.10 0.6	-0.11 0.5	0.32 0.067		
Non-obese (n=34)										

Adp- Adiponectin; Lep- Leptin; Ins- Insulin; TG- triglyceride; Glu- Glucose

Gender and age

When all cats were analyzed together, the adjusted geometric mean of serum concentrations of adiponectin was 75% higher, and the adjusted geometric mean of serum glucose concentration was 15% lower in neutered females than in neutered males (Table 9). In addition, an increase of one year of age was associated with a 24% increase in the adjusted geometric mean of serum concentrations of leptin (Table 10).

Table 9. Associations between gender and adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol in 55 healthy neutered cats.*

Variable	Ratio (95% CI)†	<i>P</i>	Partial η^2
Adiponectin	1.75 (1.08, 2.81)	0.02	0.11
Leptin	1.10 (0.94, 1.30)	0.2	0.03
Insulin	0.99 (0.76, 1.30)	1.0	0.00
Glucose	0.85 (0.76, 0.95)	0.006	0.15
Triglyceride	0.87 (0.68, 1.12)	0.3	0.03
Cholesterol	1.03 (0.86, 1.22)	0.8	0.002

* Covariates in the GLM include: gender, age, BCS, DHA, DPA, EPA, and ALA.

† Ratios (female:male) and 95% CI of adjusted geometric means of serum concentrations of each variables.

Table 10. Associations between age and adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol in 55 healthy cats.*

Variable	Fold increase (95% CI)†	<i>P</i>	Partial η^2
Adiponectin	0.76 (0.44, 1.30)	0.3	0.02
Leptin	1.24 (1.04, 1.49)	0.02	0.11
Insulin	0.91 (0.67, 1.23)	0.5	0.01
Glucose	0.91 (0.80, 1.04)	0.2	0.04
Triglyceride	0.83 (0.63, 1.10)	0.2	0.04
Cholesterol	1.08 (0.89, 1.32)	0.4	0.01

Table 10 (cont'd)

* Covariates in the GLM include: gender, age, BCS, DHA, DPA, EPA, and ALA.

† Fold increases and 95% CI in adjusted geometric means of serum concentrations of each variable with increase of 1 year of age.

n3PUFAs

In the separate analyses for the non-obese group, an increase of 1 mg/100mg FA in serum concentration of EPA was associated with a 62% decrease in the adjusted geometric mean of serum concentration of adiponectin, and a 43% increase in the adjusted geometric mean of serum concentration of leptin (Table 11). In the separate analyses for the obese group, an increase of 1 mg/100mg FA in serum concentration of EPA was associated with a 643% increase in the adjusted geometric mean of serum concentration of adiponectin, a 70% decrease in the adjusted geometric mean of serum concentration of insulin, and an 84% decrease in the adjusted geometric mean of serum concentration of triglyceride (Table 12). Additionally, an increase of 1 mg/100mg FA in serum concentration of DHA was associated with a 54% increase in the adjusted geometric mean of serum concentration of adiponectin in the separate analysis for the obese group (95% CI of fold increase of adjusted geometric mean: 1.02, 2.32; $P = 0.041$; Partial $\eta^2 = 0.22$). No significant associations were present between serum concentration of DHA and serum concentration of adiponectin in the separate analysis for the non-obese group or between concentration of DHA and leptin, insulin, glucose, triglyceride, or glucose in the separate analyses for the non-obese or obese groups.

Table 11. Associations between EPA and adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol in 34 non-obese cats.*

Variable	Mean (95% CI)†	Fold increase (95% CI)‡	P	Partial η^2
Adiponectin	2.4 µg/mL (1.8, 3.1)	0.38 (0.17, 0.83)	0.017	0.18
Leptin	4.4 ng/mL (4.1, 4.8)	1.43 (1.12, 1.83)	0.006	0.23
Insulin	34 pmol/L (29, 39)	0.95 (0.61, 1.50)	0.8	0.00
Glucose	5.7 mmol/L (5.3, 6.1)	0.97 (0.78, 1.20)	0.8	0.00
Triglyceride	65 mg/dL (58, 72)	0.84 (0.61, 1.16)	0.3	0.04
Cholesterol	128 mg/dL (117, 140)	0.87 (0.67, 1.13)	0.3	0.04

* Covariates in the GLM include: gender, age, EPA, and DHA (leptin, insulin, glucose, triglyceride, and cholesterol) or gender, EPA, and DHA (adiponectin).

†Covariates appearing in the models are evaluated at their mean values: EPA = 0.31 mg/100mg FA, DHA = 1.98 mg/100 mg FA, Age = 4.9 years.

‡ Fold increases and 95% CI in adjusted geometric means of serum concentration of each variable with an increase of 1 mg/100 mg FA in serum concentration of EPA.

Table 12. Associations between EPA and adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol in 21 obese cats.*

Variable	Mean (95% CI)†	Fold increase (95% CI)‡	P	Partial η^2
Adiponectin	1.3 µg/mL (0.8, 2.0)	7.43 (1.05, 52.30)	0.045	0.22
Leptin	5.8 ng/mL (4.8, 7.1)	0.84 (0.35, 2.01)	0.7	0.01
Insulin	46 pmol/L (36, 60)	0.30 (0.10, 0.91)	0.035	0.25
Glucose	5.1 mmol/L (4.6, 5.7)	0.98 (0.93, 1.02)	0.3	0.07
Triglyceride	97 mg/dL (71, 134)	0.16 (0.04, 0.65)	0.014	0.32
Cholesterol	137 mg/dL (116, 161)	0.96 (0.47, 1.97)	0.9	0.00

* Covariates in the GLM include: gender, age, EPA, and DHA (leptin, insulin, glucose, triglyceride, and cholesterol) or gender, EPA, and DHA (adiponectin).

† Covariates appearing in the models are evaluated at their mean values: EPA = 0.24 mg/100 mg FA, DHA = 1.92 mg/100 mg FA, Age = 4.9 years.

‡ Fold increases and 95% CI in adjusted geometric means of serum concentration of each variable with an increase of 1 mg/100 mg FA in serum concentration of EPA.

1.2.4. Associations between FAs or troglitazone treatment, body condition, or anatomic location and adipokines secretion in primary canine adipose tissue culture

Study aims

- Determine the effect of body condition on secretion of adiponectin and leptin from canine mature adipocytes and secretion of IL6 and TNF α from canine SVCs in primary culture.
- Determine the effect of adipose tissue anatomic location on secretion of adiponectin and leptin from canine mature adipocytes and secretion of IL6 and TNF α from canine SVCs in primary culture.
- Determine the effect of a PPAR γ -agonist on secretion of adiponectin and leptin from canine mature adipocytes and secretion of IL6 and TNF α from canine SVCs in primary culture.
- Determine the effect of an n3PUFA (EPA), an n6PUFA (AA), and a SFA (PA) on secretion of adiponectin and leptin from canine mature adipocytes and secretion of IL6 and TNF α from canine SVCs in primary culture.

Methods

Study design

Adipose tissue samples were collected from 16 healthy intact female dogs at the time of routine spay procedure. Body condition was evaluated using a BCS system on a 1–9 scale (Laflamme, 1997b). The study procedure was reviewed and approved by the institutional animal care and use committee at Michigan State University. Written informed consent was obtained from all owners.

Adipose tissue preparation and digestion

Subcutaneous (n=12) and mesenteric (n=16) adipose tissue samples (0.5-4.0g) were obtained aseptically and immediately placed in 15 mL of medium at 4 °C (as detailed in Appendix C, Protocol 4). Samples were transferred to the laboratory and processing started within 30 minutes. The tissue samples were digested; mature adipocytes were separated from stromovascular cells (SVCs). Aliquots of 100 mg adipocytes were each resuspended in 1 mL fresh medium in 5 mL polypropylene tubes. SVCs were resuspended in fresh medium in a final concentration of 50,000-150,000 cells/mL and aliquoted in 24-well plates (1 mL per well). Adipocytes and SVCs were incubated in a humidified incubator with 5% CO₂ at 37 °C.

Incubation and treatment

FA treatment media were prepared by dilution of stock solution in medium to a final concentration of 25 µM, 50 µM, or 100 µM. Troglitazone (Sigma, T2573) treatment medium was prepared by dilution into a final concentration of 10 µM. Control medium was prepared by

dilution of the same volume of ethanol in medium. The final concentration of ethanol was < 0.2%. Treatment and control media were incubated at 37 °C for 2 hours prior to being added to the cells. Following 24 hours of initial incubation, cells in each culture tube or well were resuspended in treatment or control medium. Following additional 48 hours of incubation, packed adipocytes volume (PAV) was determined for each tube containing adipocytes and SVC score was determined for each well (for details, see Appendix C, Protocol 4).

Medium analysis

Medium samples from cultured mature adipocytes were analyzed for concentrations of adiponectin and leptin, and samples from SVCs were analyzed for concentrations of IL6 and TNF α .

Medium concentrations of adiponectin, IL6 and TNF α were measured using commercially available assays (Canine Adiponectin ELISA, Millipore; Canine IL6 ELISA, R&D Systems; Canine TNF α ELISA, R&D Systems- See Appendix C, Protocol 1). Concentrations of leptin in medium samples from cultured mature adipocytes were nondetectable using an assay previously used with canine serum samples (Canine Leptin ELISA, Millipore- See Appendix C, Protocol 1)

Data analysis

Associations between adipose tissue anatomic location (mesenteric or subcutaneous), troglitazone treatment (troglitazone or control), or BCS and the outcome variables (medium concentrations of adiponectin, IL6, and TNF α .) were evaluated using GEE with PAV as an

additional covariate for adiponectin or SVC score as an additional covariate for IL6 and TNF α . Bonferroni correction was applied for multiple post-hoc pairwise comparisons between groups.

Associations between FA treatment (EPA, AA, PA, or control) and the outcome variables (medium concentrations of adiponectin, IL6, and TNF α) were evaluated using GEE. Due to significant interaction effects between adipose tissue anatomic location and treatment at concentration of 50 μ M and 100 μ M, separate analyses for each anatomic location (mesenteric or subcutaneous) and FAs concentration (25 μ M, 50 μ M, and 100 μ M) were performed. In addition, analysis for the two adipose tissue depots together was performed for FA treatment at 25 μ M since no interaction effect was present at this concentration. PAV and BCS were included as covariates in the analyses of adiponectin; SVC score and BCS as covariate in the analyses of IL6 and TNF α . Bonferroni correction was applied for multiple post-hoc pairwise comparisons between treatments and control. Linear correlations between PAV or SVC score and medium concentrations of adipokines were determined using Spearman correlation coefficient.

Data were analyzed using a commercially available statistic program (SPSS® 19.0 for Windows). $P \leq 0.05$ was considered statistically significant.

Results

Dogs and cultures

Dogs were of various breeds, including mixed breed (6), American Pitbull (3), German shorthair pointer (2), Beagle (2), and 1 of each of the following: Jack Russell Terrier, Boxer, and ShiTzu. Age was estimated to range from 6 to 18 months. BCS ranged from 4 to 8; Mean (SE) was 5.4 (0.3).

PAV ranged from 2% to 6%; Mean (SE) was 3.8% (0.2) in cultures from mesenteric tissue and 4.5% (0.3) in cultures from subcutaneous tissue. SVC score ranged from 1 to 5; Mean (SE) was 3.9 (0.4) in cultures from mesenteric tissue and 3.1 (0.3) in cultures from subcutaneous tissue. Medium adiponectin concentration was significantly correlated to PAV ($r = 0.64$; $P < 0.001$). Medium IL6 concentration was significantly correlated to SVC score ($r = 0.74$; $P < 0.001$). Medium TNF α was not significantly correlated to SVC score ($r = 0.15$; $P = 0.09$)

BCS, adipose tissue anatomic location, and troglitazone treatment

Adiponectin-

BCS was significantly associated with medium adiponectin concentration ($P = 0.028$). An increase of 1 unit of BCS was associated with a decrease of 2.9 ng/mL (SE: 1.3) in adjusted mean medium adiponectin concentrations.

Anatomic location of adipose tissue was significantly associated with medium adiponectin concentration ($P < 0.001$). Adjusted mean medium adiponectin concentrations from mesenteric adipocytes were 61% and 44% lower compared subcutaneous adipocytes in control and troglitazone treated cultures, respectively (Figure 4A). Individual basal medium adiponectin concentrations normalized to PAV in mesenteric and subcutaneous adipose tissue are presented in Figure 5A.

Troglitazone treatment had a significant effect on medium adiponectin concentration ($P < 0.001$). Adjusted mean medium adiponectin concentrations from troglitazone treated adipocytes were 100% and 39% higher compared to control adipocytes in mesenteric and subcutaneous cultures, respectively (Figure 4A). Individual medium adiponectin concentrations normalized to PAV in control and troglitazone treated adipocytes are presented in Figure 5B.

Interleukin-6-

BCS was significantly associated with medium IL6 concentration ($P = 0.040$). An increase of 1 unit of BCS was associated with a decrease of 9776 pg/mL (SE 4753) in adjusted mean medium IL6 concentrations.

Anatomic location of adipose tissue was significantly associated with medium IL6 concentration ($P = 0.002$). Adjusted mean medium IL6 concentrations from mesenteric SVC were 219% and 389% higher compared subcutaneous SVC in control and troglitazone treated cultures, respectively (Figure 4B). Individual basal medium IL6 concentrations normalized to SVC score in mesenteric and subcutaneous adipose tissue are presented in Figure 5C.

Troglitazone treatment had a significant effect on medium IL6 concentration ($P=0.024$). Adjusted mean medium IL6 concentrations from troglitazone treated SVC were 14% and 44% lower compared to control SVC in mesenteric and subcutaneous cultures, respectively (Figure 4B). Individual basal medium IL6 concentrations normalized to SVC score in control and troglitazone treated adipocytes are presented in Figure 5D.

Tumor Necrosis Factor- α -

BCS was significantly associated with medium TNF α concentration ($P = 0.040$). An increase of 1 unit of BCS was associated with an increase of 3.8 pg/mL (SE 1.9) in adjusted mean medium TNF α concentrations.

Anatomic location of adipose tissue was significantly associated with medium TNF α concentration ($P = 0.004$). Adjusted mean medium TNF α concentrations from mesenteric SVC were 89% and 140% higher compared subcutaneous SVC in control and troglitazone treated cultures, respectively (Figure 4C). Individual basal medium TNF α concentrations normalized to SVC score in mesenteric and subcutaneous adipose tissue are presented in Figure 5E.

Troglitazone treatment had a significant effect on medium TNF α concentration ($P = 0.004$). Adjusted mean medium TNF α concentrations from troglitazone treated SVC were 19% and 37% lower compared to control SVC in mesenteric and subcutaneous SVC cultures, respectively (Figure 4C). Individual basal medium TNF α concentrations normalized to SVC score in control and troglitazone treated adipocytes are presented in Figure 5F.

Figure 4. Medium adiponectin concentrations in primary adipocytes cultures (A) and medium IL6 (B) and TNF α (C) concentrations in SVC cultures in troglitazone-treated (■) and control (□) cultures from mesenteric and subcutaneous adipose tissue of healthy dogs.†

† Bar represents the adjusted mean medium concentration and error bar represents 1 standard error. Covariates appearing in the GEE model are fixed at their mean values as follows; for adiponectin analysis: PAV = 4.1, BCS = 5.3; for IL6 analysis: SVC score = 3.9, BCS = 5.0; for TNF α analysis: SVC score = 3.7, BCS = 5.0. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ for pairwise comparisons with Bonferroni correction for multiple pairwise comparisons.

Figure 4 (cont'd)

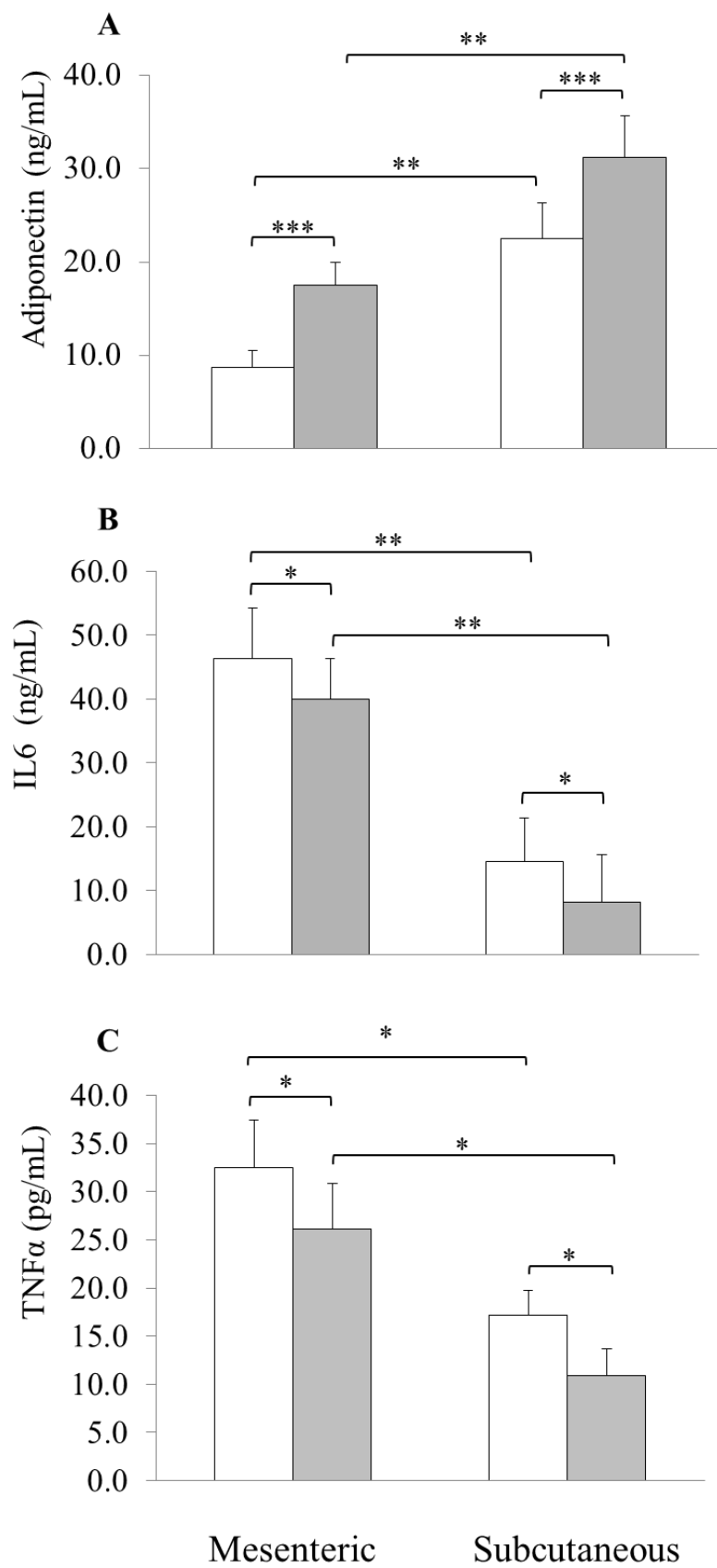
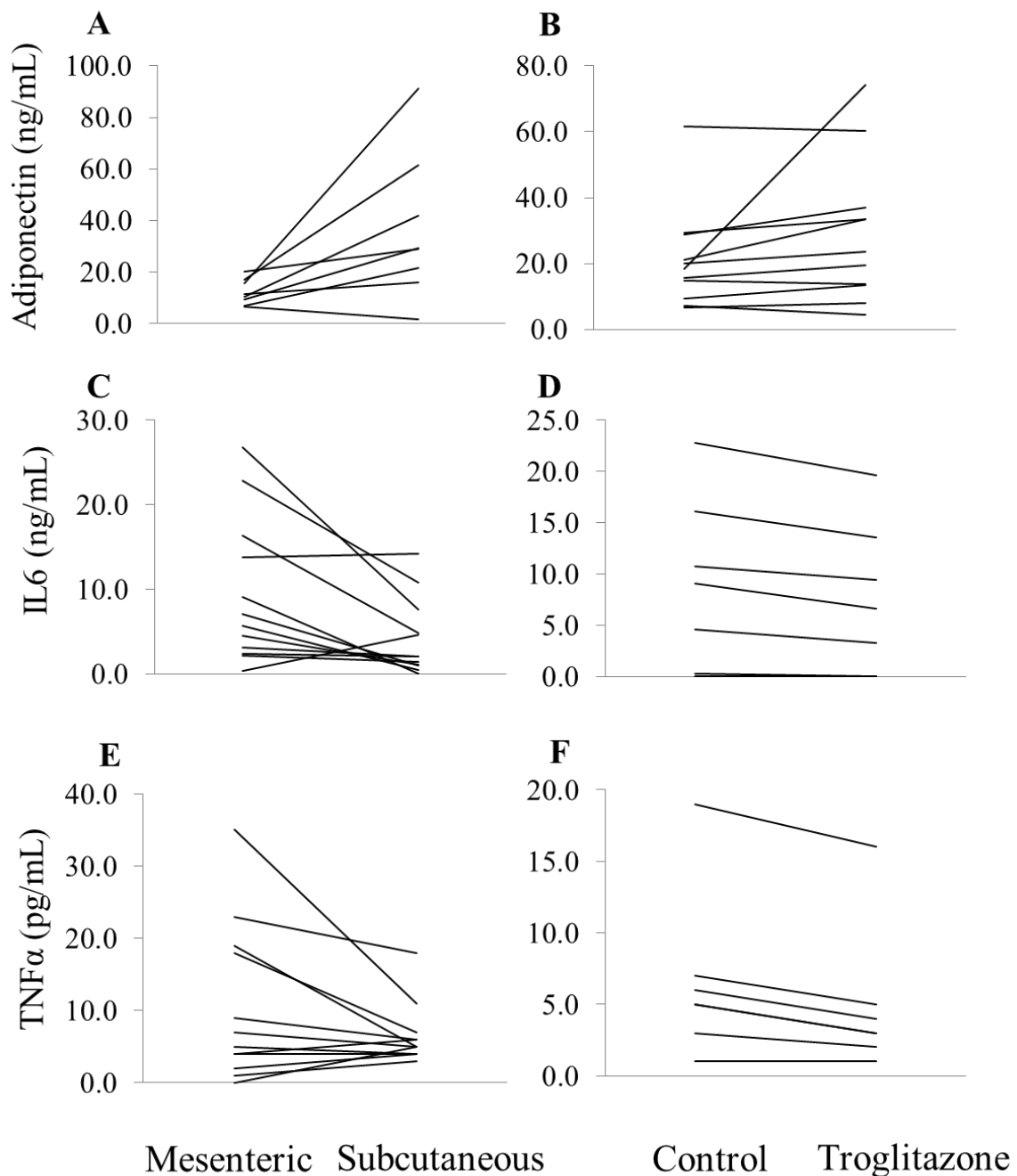


Figure 5. Medium concentrations of adiponectin in primary adipocytes cultures (A, B) and IL6 (C, D) and TNF α (E, F) in SVC cultures from mesenteric and subcutaneous adipose tissue (A, C, E) or in control and troglitazone-treated cultures (B, D, F) of healthy dogs.[†]

[†] Presented are values of individual dogs. Concentrations of adiponectin are normalized to PAV = 5 and concentrations of IL6 and TNF α are normalized to SVC score = 5.



FA treatment

Adiponectin-

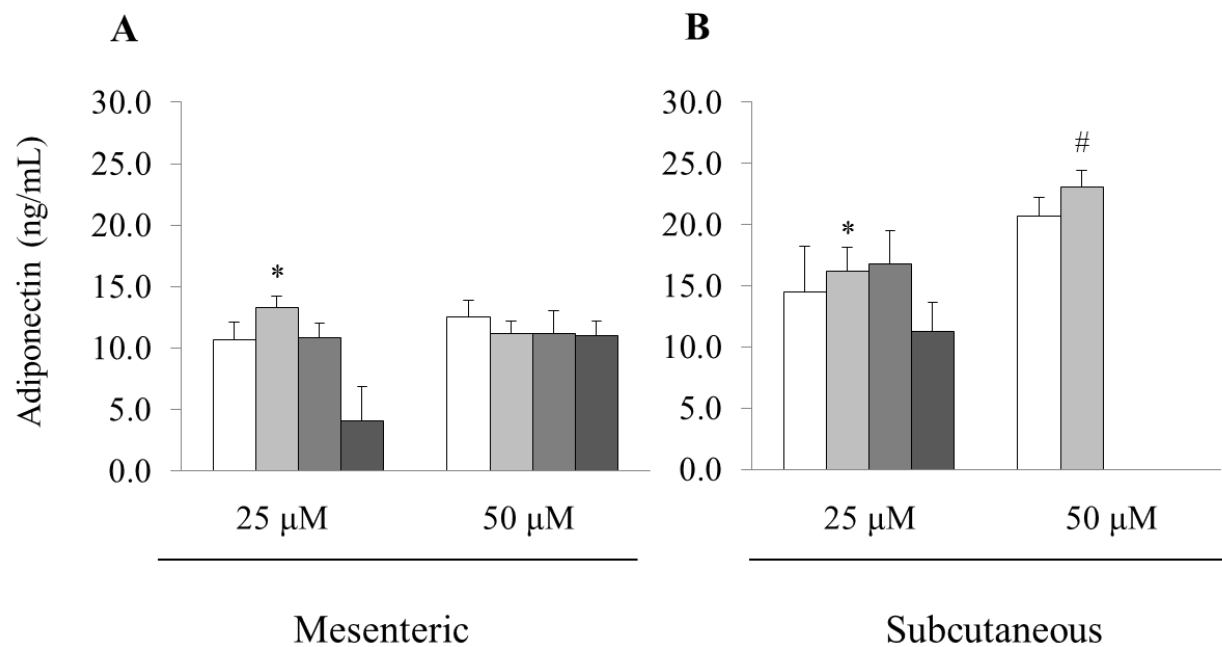
In the analysis for both anatomic locations together, FA treatment at 25 μM had a significant effect on medium adiponectin concentration ($P = 0.001$). PA treatment was associated with significantly lower adjusted medium adiponectin concentration than the control ($P = 0.042$).

In separate analyses for adipose tissue anatomic location, FA treatment at 25 μM had a significant effect on medium adiponectin concentration both in mesenteric ($P = 0.006$) and subcutaneous ($P < 0.001$) adipocytes cultures (Figure 6), but differences in adjusted medium adiponectin concentration between FA treatments and control were not significant following Bonferroni correction for multiple pairwise comparisons. EPA treatment was associated with significantly higher adjusted medium adiponectin concentration than PA both in mesenteric ($P = 0.010$) and subcutaneous ($P < 0.001$) tissues (Figure 6A and 6B).

FA treatment at 50 μM had a significant effect on medium adiponectin concentration in subcutaneous ($P < 0.001$) but not in mesenteric ($P = 0.2$) adipocytes cultures (Figure 6). In subcutaneous adipocytes culture, EPA treatment was associated with significantly ($P < 0.001$) higher adjusted medium adiponectin concentration than the control (Figure 6B).

Figure 6. Medium adiponectin concentrations in FA [(□) EPA, (▨) AA, (■) PA] -treated and control (□) primary adipocyte cultures from mesenteric (A) and subcutaneous (B) adipose tissue of healthy dogs.†

†Bar represents the adjusted mean medium concentration and error bar represents 1 standard error. Covariates appearing in the GEE model are fixed at their mean values as follows; for analysis of FA treatment at 25μM, 50μM, and 100μM in mesenteric and subcutaneous tissue: PAV = 3.9, 3.9 and 3.9, 5.5 ; BCS = 5.3, 5.0 and 6.0, 5.7. # $P < 0.001$ compared to control or * $P \leq 0.01$ compared to PA with Bonferroni correction for multiple pairwise comparisons.



Interleukin-6-

FA treatment at 25 μM did not have a significant effect on medium IL6 concentration in mesenteric ($P = 1.0$) or subcutaneous ($P = 0.1$) SVC cultures (Figure 7A and 7B).

FA treatment at 50 μM had a significant effect on medium IL6 concentration in subcutaneous ($P < 0.001$) but not in mesenteric ($P = 0.4$) SVC cultures. In subcutaneous SVC culture, EPA treatment was associated with significantly ($P = 0.003$) lower and PA treatment was associated with significantly ($P = 0.037$) higher adjusted medium IL6 concentration than the control (Figure 7B).

FA treatment at 100 μM had significant effects on medium IL6 concentration both in mesenteric ($P < 0.001$) and subcutaneous ($P < 0.001$) SVC cultures. In subcutaneous SVC culture, EPA treatment was associated with significantly ($P = 0.013$) lower and PA treatment was associated with significantly ($P < 0.001$) higher adjusted medium IL6 concentration than the control (Figure 6B). In mesenteric SVC culture, differences in adjusted medium IL6 concentration were not significant following Bonferroni correction for multiple pairwise comparisons (Figure 7A).

Tumor Necrosis Factor- α -

FA treatment in mesenteric SVC cultures had significant effects on medium TNF α concentrations at concentrations of 25 μM ($P = 0.006$), 50 μM ($P = 0.011$), and 100 μM ($P < 0.001$). Control treatment was associated with significantly higher adjusted medium TNF α concentrations than EPA, AA, and PA at 25 μM ($P = 0.009$, $P = 0.003$, and $P = 0.002$, respectively) and 50 μM ($P = 0.008$, $P = 0.004$, and $P = 0.006$, respectively). At 100 μM ,

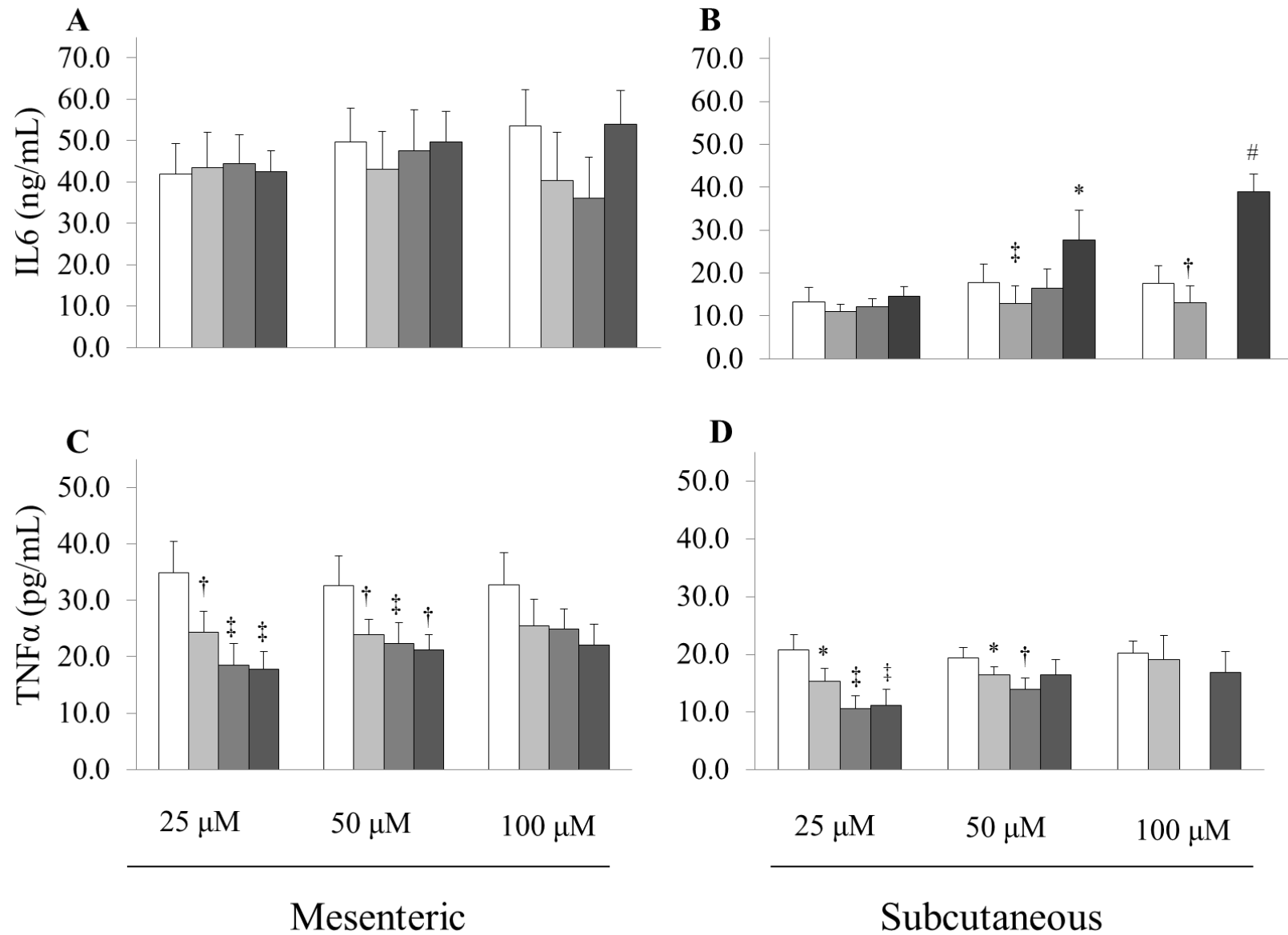
differences in adjusted medium TNF α concentration were not significant following Bonferroni correction for multiple pairwise comparisons (Figure 7C).

FA treatment in subcutaneous SVC cultures had significant effects on medium TNF α concentrations at concentrations of 25 μ M ($P = 0.002$) and 50 μ M ($P < 0.001$), but not 100 μ M ($P = 0.3$). Control treatment was associated with significantly higher adjusted medium TNF α concentrations than EPA and AA treatments at concentration of 25 μ M ($P = 0.037$ and $P = 0.001$, respectively) and 50 μ M ($P = 0.050$ and $P = 0.009$, respectively) and significantly higher adjusted concentrations than PA treatment at concentration of 25 μ M only ($P = 0.003$) (Figure 7D).

Figure 7. Medium IL6 (A, B) and TNF α (C, D) concentrations in FA [(□) EPA, (▣) AA, (■) PA] -treated and control (□) SVC cultures from mesenteric (A, C) and subcutaneous (B, D) adipose tissue of healthy dogs.[†]

[†] Bar represents the adjusted mean medium concentration and error bar represents 1 standard error. Covariates appearing in the GEE model are fixed at their mean values as follows; for analysis of FA treatment at 25 μ M, 50 μ M, and 100 μ M in mesenteric and subcutaneous tissue for IL6: SVC score = 3.9, 4.1, 4.5 and 3.2, 3.8, 3.9; BCS = 5.4, 5.0, 5.1 and 5.7, 5.2, 5.3, respectively and for TNF α : SVC score = 4.1, 4.4, 4.6 and 3.1, 3.5, 3.7; BCS = 5.3, 4.9, 5.0 and 5.7, 5.0, 5.0, respectively. * $P < 0.05$, [†] $P < 0.01$, [‡] $P < 0.005$, # $P < 0.001$ compared to control with Bonferroni correction for multiple pairwise comparisons.

Figure 7 (cont'd)



1.2.5. Associations between FAs or troglitazone treatment, body condition, or anatomic location and adipokines secretion in primary feline adipose tissue culture

Study aims

- Determine the effect of body condition on secretion of adiponectin and leptin from feline mature adipocytes and secretion of IL6 and TNF α from feline SVCs in primary culture.
- Determine the effect of adipose tissue anatomic location on secretion of adiponectin and leptin from feline mature adipocytes and secretion of IL6 and TNF α from feline SVCs in primary culture.
- Determine the effect of a PPAR γ -agonist on secretion of adiponectin and leptin from feline mature adipocytes.
- Determine the effect of an n3PUFA (EPA), an n6PUFA (AA), and a SFA (PA) on secretion of adiponectin and leptin from feline mature adipocytes and secretion of IL6 and TNF α from feline SVCs in primary culture.

Methods

Study design

Adipose tissue samples were collected from 18 healthy intact mixed breed female cats at the time of routine spay procedure. Body condition was evaluated using a body condition scoring (BCS) system on a 1–9 scale (Laflamme, 1997a). The study procedure was reviewed and approved by the institutional animal care and use committee at Michigan State University. Written informed consent was obtained from all owners.

Adipose tissue preparation and digestion

Mesenteric (n=18) and subcutaneous (n=17) adipose tissue samples (0.5-4.0g) were obtained aseptically and immediately placed in 15 mL of medium at 4 °C (as detailed in Appendix C, Protocol 4). Samples were transferred to the laboratory and processing started within 30 minutes. The tissue samples were digested; mature adipocytes were separated from SVCs. Aliquots of 100 mg adipocytes were each resuspended in 1 mL fresh medium in 5 mL polypropylene tubes. SVC were resuspended in fresh medium at a final concentration of 50,000-150,000 cells/mL and aliquoted in 24-well plates (1 mL per well). Adipocytes and SVC were incubated in a humidified incubator with 5% CO₂ at 37 °C.

Incubation and treatment

FAs treatment media were prepared by dilution of stock solutions in medium to a final concentration of 25µM, 50µM, or 100µM. Troglitazone (Sigma, T2573) treatment medium was prepared by dilution into a final concentration of 10µM. Control medium was prepared by

dilution of the same volume of ethanol in medium. The final concentration of ethanol was < 0.2%. Treatment and control media were then incubated at 37 °C for 2 hours prior to being added to the cells. Following 24 hours of initial incubation, cells in each culture tube or well were resuspended in treatment or control medium. Following additional 48 hours of incubation, PAV was determined for each tube containing adipocytes and SVC score was determined for each well (for details, see Appendix C, Protocol 4).

Medium analysis

Medium samples from cultured mature adipocytes were analyzed for concentrations of adiponectin and leptin and samples from SVCs were analyzed for concentrations of IL6 and TNF α .

Medium concentrations of adiponectin, IL6 and TNF α were measured using commercially available assays validated in our laboratory (Mouse/rat Adiponectin ELISA, B-Bridge; Feline IL6 ELISA, R&D Systems; Feline TNF α ELISA, R&D Systems- See Appendix C, Protocol 1). Concentrations of leptin were nondetectable using an assay previously used with canine serum samples (Multispecies Leptin RIA, Millipore- See Appendix C, Protocol 2)

Data analysis

Associations between adipose tissue anatomic location (mesenteric or subcutaneous), BCS, or troglitazone treatment (troglitazone or control) and concentration of adiponectin were evaluated using GEE with PAV as an additional covariate. Associations between adipose tissue anatomic location (mesenteric or subcutaneous) or BCS and concentrations of IL6 and TNF α were evaluated using GEE with SVC score as an additional covariate. Associations between FA

treatment (EPA, AA, PA, or control) and medium concentrations of adiponectin were evaluated using GEE with BCS, adipose tissue anatomic location and PAV as covariates. Due to significant interaction effects between adipose tissue anatomic location and FA treatments associations in the analyses of the associations with medium concentrations of IL6 and TNF α , separate analyses were performed for each anatomic location and FAs concentration (25 μ M, 50 μ M, and 100 μ M) with BCS and SVC score as covariates. Bonferroni correction was applied for multiple post-hoc comparisons between treatments and control. Linear correlations between PAV or SVC score and medium concentrations of adipokines were determined using Spearman correlation coefficient.

Data were analyzed using a commercially available statistic program (SPSS® 19.0 for Windows). $P \leq 0.05$ was considered statistically significant.

Results

Cats and cultures

Age was estimated to range from 6 to 18 months. BCS ranged from 5 to 7; Mean (SE) was 5.5 (0.1). PAV ranged from 2% to 8%; Mean (SE) was 4.9% (0.1) in cultures from mesenteric tissue and 4.4% (0.2) in cultures from subcutaneous tissue. SVC score ranged from 1 to 5; Mean (SE) was 4.1 (0.1) cultures from mesenteric tissue and 3.5 (0.1) in cultures from subcutaneous tissue. Medium adiponectin concentration was significantly correlated to PAV ($r = 0.52$; $P < 0.001$). Medium IL6 concentration was significantly correlated to SVC score ($r = 0.54$; $P < 0.001$) and medium TNF α tended to correlate to SVC score ($r = 0.33$; $P = 0.055$).

BCS, adipose tissue anatomic location, and troglitazone treatment

BCS was not significantly associated with medium concentrations of adiponectin ($P = 0.4$), IL6 ($P = 0.1$), or TNF α ($P = 0.8$).

Adipose tissue anatomic location was significantly associated with medium concentrations of IL6 ($P = 0.003$), but not TNF α ($P = 0.2$) or adiponectin ($P=0.8$) (Figures 8 and 9). Adjusted mean medium IL6 concentration from mesenteric SVC was 153% higher compared to subcutaneous SVC cultures.

Troglitazone treatment did not have a significant effect on medium adiponectin concentration ($P = 0.4$) (Figure 9).

Figure 8. Medium IL6 and TNF α concentrations in control SVC cultures from mesenteric and subcutaneous adipose tissue of healthy cats.†

† Bar represents the adjusted mean medium concentration and error bar represents 1 standard error. Covariates appearing in the GEE models are fixed at their mean values as follows; SVC score = 3.7, BCS = 5.5. * $P = 0.003$.

Figure 8 (cont'd)

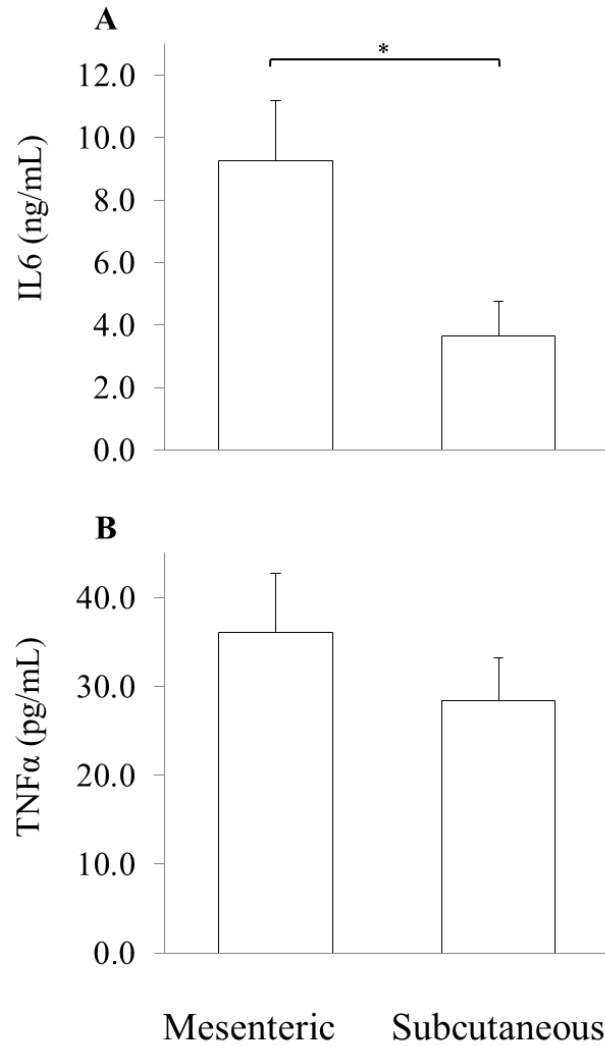
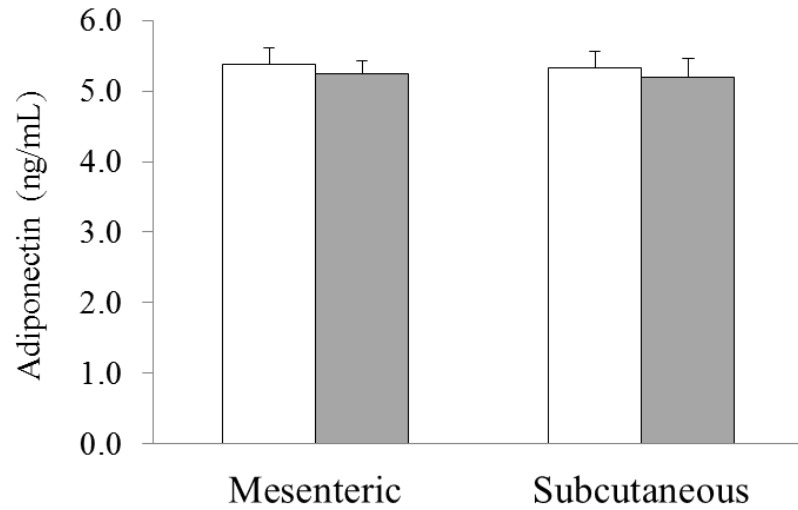


Figure 9. Medium adiponectin concentrations in troglitazone-treated (■) and control (□) primary adipocytes cultures from mesenteric and subcutaneous adipose tissue of healthy cats.†

† Bar represents the adjusted mean medium concentration and error bar represents 1 standard error. Covariates appearing in the GEE model are fixed at their mean values as follows; PAV = 4.2, BCS = 5.6.

Figure 9 (cont'd)



FA treatment

Adiponectin-

FA treatments did not have significant effects on medium adiponectin concentrations in FA concentration of 25 μ M ($P = 0.2$), 50 μ M ($P = 0.8$), or 100 μ M ($P = 0.7$).

Interleukin 6-

FA treatment at 25 μ M, 50 μ M, and 100 μ M had significant effects on medium IL6 concentration both in mesenteric ($P = 0.029$, $P < 0.001$, and $P < 0.001$, respectively) and subcutaneous ($P = 0.007$, $P < 0.001$, and $P < 0.001$, respectively) SVC cultures. AA treatment was associated with significantly higher medium IL6 concentration than the control at 25 μ M, 50 μ M, and 100 μ M in subcutaneous ($P = 0.05$, $P = 0.002$, and $P < 0.001$, respectively) and mesenteric ($P = 0.034$, $P = 0.001$, and $P < 0.001$, respectively) SVC cultures (Figure 10A and B).

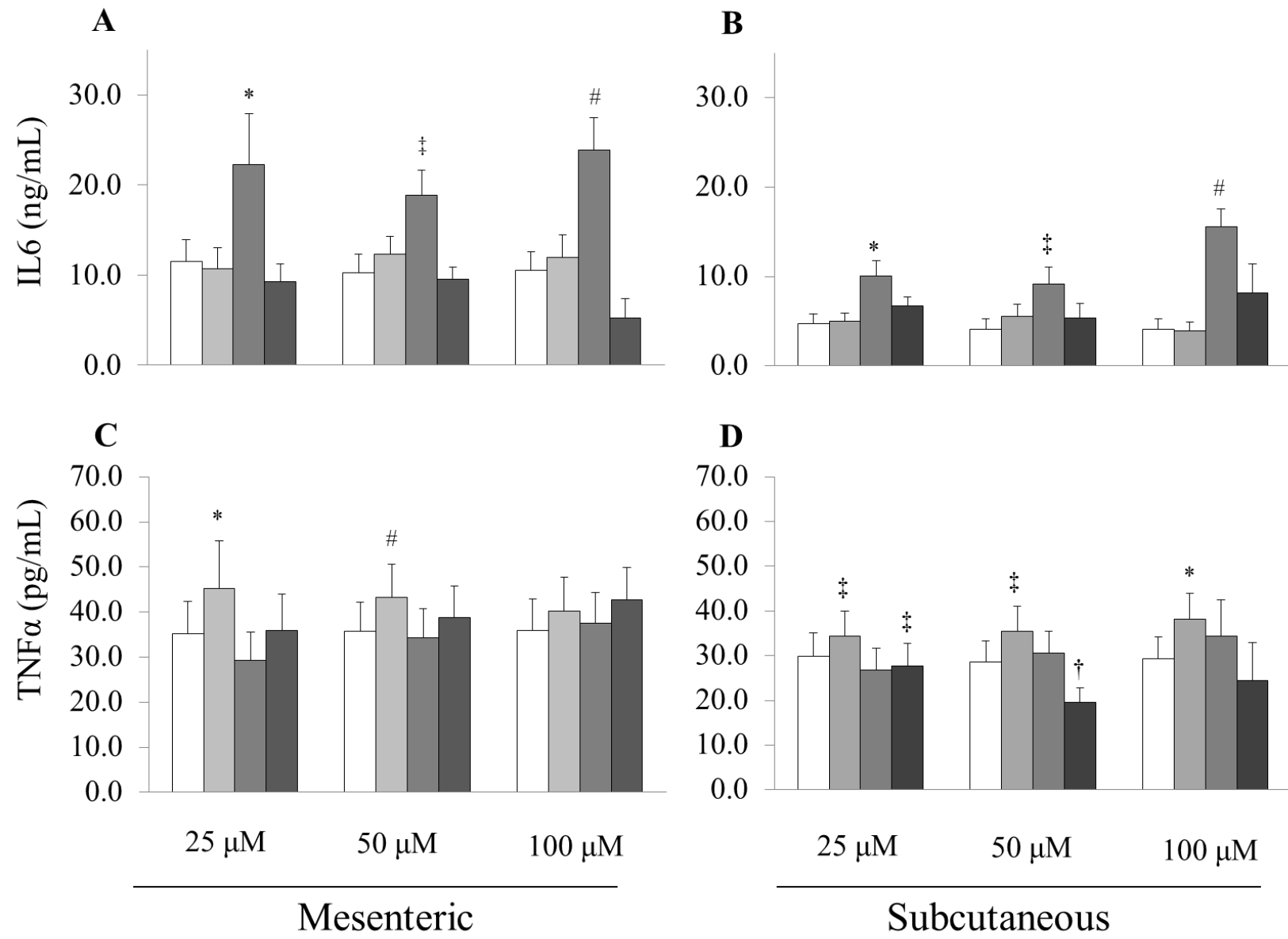
Tumor Necrosis Factor α -

FA treatment at 25 μ M and 50 μ M had significant effects on medium TNF α concentrations in mesenteric ($P = 0.009$ and $P < 0.001$, respectively) and subcutaneous ($P < 0.001$ and $P < 0.001$, respectively) SVC cultures. At 100 μ M, FA treatment had a significant effect on medium TNF α concentrations in subcutaneous ($P < 0.001$) but not in mesenteric ($P = 0.1$) SVC cultures. AA treatment at 25 μ M was associated with significantly lower adjusted medium TNF α concentrations than control in mesenteric ($P = 0.032$) SVC cultures. EPA treatment was associated with significantly higher adjusted medium TNF α concentrations than control in subcutaneous SVC cultures at 25 μ M, 50 μ M, and 100 μ M ($P = 0.002$, $P = 0.001$, and $P = 0.015$, respectively) and in mesenteric SVC cultures at 50 μ M ($P < 0.001$). PA treatment was associated with significantly lower adjusted medium TNF α concentrations than control in subcutaneous SVC cultures at 25 μ M and 50 μ M ($P = 0.001$ and $P = 0.008$, respectively) (Figure 10C and D).

Figure 10. Medium IL6 (A, B) and TNF α (C, D) concentrations in FA [(□) EPA, (■) AA, (■) PA]-treated and control (□) SVC cultures from mesenteric (A, C) and subcutaneous (B, D) adipose tissue of healthy cats.†

† Bar represents the adjusted mean medium concentration and error bar represents 1 standard error. Covariates appearing in the GEE model are fixed at their mean values as follows; for analysis of FA treatment at 25 μ M, 50 μ M, and 100 μ M in mesenteric tissue and subcutaneous tissue for IL6: SVC score = 4.2, 3.9, 4.0 and 4.1, 3.5, 3.5; BCS = 5.3, 5.4, 5.5 and 5.5, 5.4, 5.4, respectively and for TNF α : SVC score = 4.2, 3.9, 4.0 and 3.8, 3.3, 3.5; BCS = 5.3, 5.4, 5.5 and 5.5, 5.5, 5.5, respectively. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.005$, # $P < 0.001$ compared to control with Bonferroni correction for multiple pairwise comparisons.

Figure 10 (cont'd)



1.3. Discussion

1.3.1. *Introductory comments*

The effects of n3PUFAs on concentrations of adipokines, as well as the potential confounders of these effects (i.e. body condition, gender, and age) were determined in circulation and/or in cell culture medium. Circulating concentrations of any adipokine may be altered by various factors and are the result of summation of expression level and production, and protein degradation and clearance rates. Moreover, adipokines may be expressed in one or multiple types of cells within the adipose tissue and possibly other organs and tissues. On the other hand, the adipose tissue culture system used in the studies reported here, allowed determination of secretion of adipokines from two separate fractions of the adipose tissue (i.e. mature adipocytes and SVCs) with exclusion of potential interactions between these fractions. Clearly, potential effects of other tissues were not involved in determining the secreted concentrations of adipokines in the culture system.

Adipokines are produced and secreted by multiple cell types within the adipose tissue. Adiponectin is synthesized and secreted exclusively by mature adipocytes (Eisele et al., 2005), whereas other cytokines, including IL6 and TNF α , are produced predominantly by the SVCs (Fain et al., 2004a; Fain et al., 2004b), although some production by mature adipocytes occurs (Eisele et al., 2005). Various types of cells within the stromovascular fraction may participate in adipose tissue inflammatory response, but the macrophages are likely to be the major contributors to secretion of inflammatory cytokines (Weisberg et al., 2003).

The degree of adipokines secretion from adipose tissue depends on multiple factors. Adiponectin production is higher in adipocytes of smaller size and with higher levels of expression and activation of PPAR γ (Maeda et al., 2001; Bouskila et al., 2005). Secretion of the

inflammatory cytokines increases with increasing numbers of macrophages within the adipose tissue and decreases with higher levels of macrophage expression and activation of PPAR γ (Jiang et al., 1998). Moreover, in vitro studies demonstrate interplay between secretion of adiponectin and inflammatory cytokines. On one hand, overexpression of adiponectin in mouse adipocytes cell line culture decreased IL6 and TNF α expression (Zhou et al., 2007), and on the other, TNF α and IL6 treatment of cultured human adipose tissue explants decreased adiponectin expression (Bruun et al., 2003). Reduction in adiponectin expression and secretion in response to TNF α has been recently reported in differentiated canine adipocytes as well (Ryan et al., 2010).

In the studies utilizing adipose tissue cells culture reported here, concentrations of adipokines were adjusted to the volume of adipocytes (adiponectin) or the number of SVCs (IL6 and TNF α). Therefore, the number of adipocytes is expected to be the same in a constant volume of adipocytes for cultures from the same tissue sample; however, some variation is possible between tissues if the size of adipocytes between those tissues is markedly different. In addition, the number of macrophages is expected to be the same for a constant number of SVCs for cultures from the same tissue sample, but variation is expected between tissues if percentage of macrophages within the total SVCs is markedly different between those tissues. Moreover, the potential reciprocal effects among adiponectin and the inflammatory cytokines are expected to have a minimal effect in the present study as the mature adipocytes and SVCs were cultured separately, although some effect of inflammatory cytokines secreted from mature adipocytes on adiponectin secretion is possible.

The methods used in these studies did not allow the measurement of concentrations of leptin secreted from mature adipocytes in canine or feline cultures, as the leptin concentrations secreted into the medium were likely below the sensitivity limit of the assays used. The

concentration of adipocytes in the culture medium (100 mg / 1 mL) was suitable for the measurement of secreted adiponectin, but was not sufficient for the measurement of secreted leptin. This was not completely unexpected, as leptin is known to circulate in much lower concentrations (ng/mL) compared to adiponectin ($\mu\text{g/mL}$). Using a 1000-times more concentrated adipocytes culture was considered unlikely to be feasible due to insufficient nutrient supply by the medium to the adipocytes at such a high concentration. This obstacle may potentially be overcome in the future by development of a highly sensitive assay for the measurement of leptin in dogs and cats, similar to the assay used with cultured human adipocytes medium (Li et al., 2008).

Measurement of circulating concentrations of inflammatory cytokines was available for a limited number of dogs (Study 1.2.1) and for none of the cats in the present studies. Moreover, concentrations were non-measurable in some of these serum samples. This is in agreement with reports from healthy human subjects (Yu and Ginsberg, 2005) as well as dogs, where plasma $\text{TNF}\alpha$ concentrations were above the detectable limit in 42% obese and only 12% lean dogs (German et al., 2009).

1.3.2. Effect of body condition on adipokines

Adiponectin and Leptin

Initial analyses of the associations of circulating concentrations of adipokines with measures of body condition (%BF) revealed significant positive correlations with leptin and significant negative correlations with adiponectin both in dogs (study 1.2.1) and cats (study 1.2.3). These findings are in agreement with other reports in dogs (Ishioka et al., 2006; Gayet et al., 2007) and cats (Hoenig et al., 2007; Ishioka et al., 2009; Muranaka et al., 2011).

The association between %BF and leptin was stronger compared to its association to adiponectin in dogs ($r = 0.78$ and $r = -0.46$, respectively), while these associations were of comparable strength in cats ($r = 0.57$ and $r = -0.55$, respectively). Similar findings of a weaker association of body condition to adiponectin compared to leptin have been previously reported in dogs (Ishioka et al., 2006). Moreover, recent studies in dogs reported lack of association between body condition and adiponectin concentrations (German et al., 2009; Mitsuhashi et al., 2010; Verkest et al., 2011a) or expression (Grant et al., 2011). When the associations between body condition and concentrations of adipokines were evaluated with consideration of additional potential influencing factors in dogs in the present study, body condition remained a strong determinant of leptin concentrations, accounting for 50% of the variability in leptin in that study, but the association with adiponectin was no longer significant. A recent study in cats (Tan et al., 2008) also reported lack of association between body condition and adiponectin concentrations. The presence of interaction effects between body condition and the additional factors in cats in

the present study precluded evaluation of the effect of body condition on concentrations of adipokines with consideration of these potential confounders

The discussion above indicates that body condition is a stronger determinant of circulating concentrations of leptin than adiponectin, at least in dogs; therefore, contradictory results regarding the association between adiponectin and body condition are reported. It is possible that the strength or even the existence of this association may be dependent on other potential factors that may have effects on concentrations of adiponectin. One such factor is concentrations of n3PUFAs. This idea is further exemplified by the findings of significant correlations between leptin and body condition both at baseline and after 30 days of fish oil supplementation in healthy dogs (Study 1.2.2), but a nearly significant correlation between adiponectin and body condition only at baseline and not following supplementation, when circulating concentrations of n3PUFAs were relatively high, potentially having a larger contribution to concentrations of adiponectin.

When dogs (Study 1.2.1) and cats (Study 1.2.3) were categorized into non-obese (BCS=4-6) and obese (BCS=7-9), associations of body condition and leptin were revealed both in the obese and non-obese groups of dogs and cats (although the association in non-obese cats was only nearly significant). Associations of body condition and adiponectin, on the other hand, were found in the obese but not the non-obese cats, and in the non-obese but not the obese dogs. Since the numbers of dogs and cats in the obese groups were smaller than the numbers in the non-obese groups, it is possible that the lack of significant association in obese dogs is due to a smaller sample size; the lack of significant association in non-obese cats, however, cannot be attributed to insufficient sample size. These findings again show the less consistent association of adiponectin concentrations to body condition compared to the association with leptin. The

presence of a significant association in obese cats only may suggest that the effect of body fat content on the concentration of adiponectin becomes more prominent above a certain threshold of adiposity.

Concentrations of adiponectin secreted from mature adipocytes into the culture medium were significantly negatively related to body condition of the adipose tissue donor in dogs (Study 1.2.4), but no association was revealed in cats (Study 1.2.5). Most adipose tissue donors included in these studies were lean; mean BCS of the dogs and cats were 5.4 and 5.5, respectively. Therefore, these findings are consistent with the associations between body condition and circulating concentrations of adiponectin discussed above, where a significant association was revealed in lean dogs (Study 1.2.1) but not in lean cats (Study 1.2.3).

Interleukin-6 and Tumor Necrosis Factor- α

A significant positive association between body condition and concentrations of TNF α secreted by SVCs to the medium was revealed in canine cultures (Study 1.2.4), but no association was present with circulating serum concentrations in dogs (Study 1.2.1). BCS was found to be negatively correlated to concentrations of IL6 both in serum (Study 1.2.1) and medium (Study 1.2.4) samples in dogs, while no associations between body condition and concentrations of IL6 or TNF α secreted to the medium were revealed in feline cultures (Study 1.2.5).

The findings of a direct relationship between medium concentrations of TNF α and BCS in dogs are consistent with the reported increase in TNF α concentration in response to overfeeding (Gayet et al., 2004) as well as its reduction with weight loss in obese dogs (German

et al., 2009). Moreover, in agreement with the present study, secretion of TNF α from human adipose tissue explants was directly related to the tissue donors' body condition (Fain et al., 2004a). In contrast, a lack of association of adipose tissue expression of adiponectin and TNF α to %BF was recently reported in healthy male dogs (Ryan et al., 2010). These conflicting findings may be explained by study design or gender differences, as female dogs were included in the study reported here while males were included in the other study.

Unlike the findings for TNF α , the inverse relationship between BCS and circulating (Study 1.2.1) and secreted (Study 1.2.4) concentrations of IL6 is in contrast to previous studies in human subjects, reporting direct relationship between circulating concentrations of IL6 and measures of body condition (Vozarova et al., 2001; Browning et al., 2008) as well as a decrease in circulating concentrations of IL6 following weight loss (Belza et al., 2009). In addition, a lack of association of %BF to adipose tissue expression of IL6 was recently reported in healthy dogs (Ryan et al., 2010). The lack of association of circulating concentrations of TNF α may be due to insufficient sample size, but may also reflect a gender effect since both males and females were included.

Potential mechanisms

Various processes that occur within the adipose tissue as it expands during obesity may affect the production and secretion of adipokines. Reduction in adipose tissue PPAR γ expression has been demonstrated in obese dogs (Gayet et al., 2007), similar to findings in humans and rodent models (Lefebvre et al., 1998). In addition, the numbers of macrophages increase in adipose tissue in obesity (Weisberg et al., 2003). These changes may result in decreased

production of adiponectin by the adipocytes and increased production of TNF α by the adipose tissue macrophages in obesity consistent with the findings of the present study. The inverse relationship of secreted IL6 to BCS cannot be simply explained in view of conflicting findings in other species as well as the opposite findings for TNF α . A potential gender difference is possible, as female dogs were included in the present study and male dogs were included in a previous study that reported no association of IL6 to %BF (Ryan et al., 2010). A gender effect on the relationship between circulating IL6 concentrations and body fat in human subjects was reported in one study, with increased concentrations found in males only (Popko et al., 2010). Additional potential factors may include species or age and activity level differences, as the dogs in the present study were younger and potentially more active relative to the population of human subjects in previous studies that reported a positive association of IL6 to measures of body condition (Vozarova et al., 2001; Browning et al., 2008). In addition, a recent study showed that acute hyperinsulinemia raised plasma IL6 concentrations in humans and that this effect was inversely associated with body condition measures (Ruge et al., 2009). The effects of feeding and hyperinsulinemia on IL6 in dogs have not been reported. However, a similar effect in the dogs in the present study could potentially lead to a larger stimulatory post prandial effect on IL6 in dogs with lower BCS, and consequently higher medium concentrations. Although the dogs were fasted for 12 hours prior to the procedure, it is possible that a longer fast is required to avoid a post prandial effect on IL6.

The lack of the expected associations between BCS and medium concentrations of IL6 or TNF α in cats in the present study may be due to the relatively narrow range of BCSs among the cats; most cats were in optimal body condition and only 1 had a BCS of 7. Therefore, minimal obesity-related pathologic changes affecting adipokines secretion were probably present in the

adipose tissue of these cats. TNF α protein and mRNA expressions levels were previously reported be higher in adipose tissue of obese compared to lean cats (Miller et al., 1998; Hoenig et al., 2006) and expression of IL6 was demonstrated to increase following ad libidum feeding and weight gain (Belsito et al., 2009).

Table 13. A summary of associations between body condition and adipokines concentrations in serum (A) and medium (B).

A.

	Dogs	Obese cats	Non-obese cats
Adiponectin	?↓	↓	N
Leptin	↑↑	↑	N
Interleukin -6	↓	-	-
Tumor Necrosis Factor - α	N	-	-

B.

	Dogs	Cats
Adiponectin	↓	N
Interleukin -6	↓	N
Tumor Necrosis Factor - α	↑	N

↑: significant positive association; ↓: significant negative association; N: no significant association; -: data unavailable

1.3.3. *Effect of gender on adipokines*

Gender accounted for 11% of the variability in circulating concentrations of adiponectin in cats (Study 1.2.3), but no association was revealed in dogs (Study 1.2.1), and no effect of gender on circulating concentrations of leptin in dogs or cats was found in these studies. The inclusion of intact female animals alone as adipose tissue donors precluded the evaluation of the effect of gender on secreted adipokines in the culture studies (Studies 1.2.4 and 1.2.5; Table 14).

Adiponectin

Higher circulating concentrations of adiponectin were found in female compared to male cats, in agreement with the gender effect on adiponectin reported in humans (Nishizawa et al., 2002) but in contrast to a previous report in cats (Tan et al., 2011). A direct effect of testosterone to decrease adiponectin secretion has been proposed as the primary factor for this gender difference in humans and rodent models (Nishizawa et al., 2002; Xu et al., 2005), but cannot explain the finding in the population of neutered cats in the current study. A recent study in a group of male and female human subjects of similar age and body condition demonstrated an inverse association of adiponectin with nonesterified FAs in men but not in women, suggesting that additional factors may be involved in the effect of gender on adiponectin concentrations (Plaisance et al., 2009). Further evaluation of potential gender differences other than sex hormones that may affect adiponectin production in neutered cats is warranted. The lack of effect of gender on circulating concentrations of adiponectin in dogs is in agreement with previous reports in dogs (Ishioka et al., 2006) and is most likely due to the fact that most dogs were neutered. Studies in dog and cats to date did not reveal gender difference in adiponectin. This

could potentially be explained by the inclusion of mostly neutered animal in some of the studies (Ishioka et al., 2006). In addition, a recent study reported no long-term change in serum concentrations of adiponectin in Beagle dogs following orchidectomy (TvariJonaviciute et al., 2011).

Leptin

No gender difference in leptin concentrations was revealed in dogs and cats in the present study, in agreement with previous studies (Ishioka et al., 2002b; Ishioka et al., 2007). In contrast, neutering with weight maintenance in cats was shown to increase circulating concentrations of leptin in males but did not alter concentrations in females (Hoenig and Ferguson, 2002). Another study demonstrated a decrease in adipose tissue mRNA expression of leptin in female cats following neutering and food restriction to maintain body weight (Belsito et al., 2009). Studies in humans and rats demonstrated an effect of estradiol to elevate and an effect of testosterone to decrease leptin concentrations (Behre et al., 1997; Shimizu et al., 1997). The absence of gender effect in the present studies in is likely due to fact that most dogs and all cats were neutered.

Table 14. A summary of associations between gender and adipokines concentrations in serum.

	Dogs	Cats
Adiponectin	Female > Male	N
Leptin	N	N

N: no significant association

1.3.4. *Effect of age on adipokines*

Age was found to have an independent effect to increase circulating concentrations of leptin both in dogs and in cats and to decrease circulating concentrations of adiponectin in dogs, but not in cats (Studies 1.2.1 and 1.2.3). Age accounted for 20% of the variability in adiponectin concentrations in dogs and 18% and 11% of the variability in leptin concentrations in dogs and cats, respectively. The inclusion of young animals only as adipose tissue donors precluded the evaluation of the effect of age on secreted adipokines in the culture studies (Studies 1.2.4 and 1.2.5; Table 15).

Leptin

Similar to the findings in the present studies, a previous study in dogs reported direct relationship between age and leptin concentrations (Ishioka et al., 2002b). This association was thought to be due to the lesser content of visceral fat in puppies younger than 1 year old. This cannot explain the association found in the present study since only dogs older than 1 year were included. A similar effect of age to increase leptin concentration was previously reported in rodent models as well (Escriva et al., 2007; Carrascosa et al., 2009) and was thought to result from decreased leptin signaling in the hypothalamus with aging and development of leptin resistance.

Adiponectin

Reports regarding the effect of age on concentrations of adiponectin in other species are conflicting. In humans and rodent models, concentrations of adiponectin were found to either not change (Yamamoto et al., 2002; Escriva et al., 2007) or to increase (Daimon et al., 2003; Isobe et al., 2005; Karakelides et al., 2010) with age. The human subjects in the later studies were older and the increase in concentrations of adiponectin was suggested to result from decreased renal function with aging (Isobe et al., 2005). This may provide at least a partial explanation for the different findings in the dogs in the present study as only 8 dogs were older than 10 years. Another study revealed an interaction of gender and age, where adiponectin concentrations increased with age in males but not in females (Adamczak et al., 2005). Such an interaction was not present in the present studies in dogs or cats.

Table 15. A summary of associations between age and adipokines concentrations in serum.

	Dogs	Cats
Adiponectin	↓	N
Leptin	↑	↑

↑: significant positive association; ↓: significant negative association; N: no significant association

1.3.5. Effect of adipose tissue anatomic location on adipokines

Adipose tissue anatomic location had an independent effect on secreted concentrations of adiponectin, IL6, and TNF in canine adipose tissue culture (Study 1.2.4) and on secreted concentrations of IL6 in feline culture (Study 1.2.5). Mean concentrations of adiponectin secreted from canine mesenteric adipose tissue were 61% lower and mean concentrations of IL6 and TNF α were 219% and 89% higher, respectively compared to concentrations secreted from subcutaneous adipose tissue. Mean IL6 concentrations secreted from feline mesenteric adipose tissue were 153% higher compared to subcutaneous tissue (Table 16).

The findings of higher secretion of adiponectin and lower secretion of IL6 and TNF α in subcutaneous compared to mesenteric tissue are in agreement with previous findings in humans (Fried et al., 1998; Fisher et al., 2002; Fain et al., 2004b; Lihn et al., 2004; Fain, 2006; Hernandez-Morante et al., 2007; Drolet et al., 2009). In contrast, a recent study in 6 healthy male dogs reported no significant difference in quantitative gene expression of adiponectin, IL6, or TNF α among different tissue depots including omental and subcutaneous fat (Ryan et al., 2010). The discrepancy between this report on gene expression and the findings on protein secretion in the present study is unclear. A gender difference cannot be ruled out as only females were included in the present study as opposed to males alone in the previous study. However, a gender effect has not been reported in other species. It is possible that the sample size in the previous report was not sufficient to reveal an existing difference.

Studies in human and animals reveal several biological and genetic differences between intraabdominal visceral-fat and peripheral subcutaneous-fat that may result in differential

secretion of adipokines. It has been demonstrated that SVCs are more numerous in visceral than in subcutaneous adipose tissue in obese human subjects (Bastelica et al., 2002; Fain et al., 2004b). A similar difference in the dogs in the present study would provide a mechanism for higher secretion of adiponectin and lower secretion of IL6 and TNF α in mesenteric compared to subcutaneous adipose tissue. In addition, expression of PPAR γ was lower in visceral compared to subcutaneous fat in non-obese human subjects (Lefebvre et al., 1998). Studies in dogs demonstrated a significant decrease in PPAR γ expression in visceral but not subcutaneous adipose tissue following induction of weight gain (Gayet et al., 2007). Potentially lower PPAR γ expression in the mesenteric compared to subcutaneous adipose tissue in the dogs in the present study would result in decreased adiponectin secretion from mature adipocytes and increased secretion of IL6 and TNF α from macrophages. A similar mechanism may explain the lower secretion of IL6 from subcutaneous compared to mesenteric adipose tissue in cats.

It is interesting to note, that although differences in the basal concentrations of adipokines between subcutaneous and mesenteric adipose tissue were present in dogs, the effect of BCS on these adipokines and the response to troglitazone were generally the same in the two depots. Comparison of the effect of troglitazone on adiponectin between subcutaneous and visceral adipose tissue in humans yielded conflicting results, with one study revealing an effect of troglitazone in the subcutaneous depot only (Phillips et al., 2008) and another study in the visceral depot only (Motoshima et al., 2002). Findings in dogs in the present study indicate an effect of troglitazone both in subcutaneous and mesenteric adipose tissue with a potentially larger effect on adiponectin in the mesenteric depot. The effect of troglitazone may have been more potent on the mesenteric adipocytes due to their lower basal expression of PPAR γ and the ability of troglitazone to up-regulate this nuclear receptor (Davies et al., 2002).

The lack of the significant differences in secretion of adiponectin and TNF α between cultures of mesenteric and subcutaneous adipose tissue in cats may be due to the relatively narrow range of BCSs among the cats in the present study; most cats were in optimal body condition and only 1 had a BCS of 7. Therefore, minimal obesity-related pathologic changes that may differentially affect adipokines secretion from mesenteric and subcutaneous adipose tissue were probably present. Interestingly, one study in cats reported higher mRNA expression of PPAR γ in visceral compared to subcutaneous adipose tissue (Zini et al., 2009). These results are in contrast to findings in humans and rodent models and may indicate a species difference that may partially explain the different results in cats.

These findings suggest that visceral fat is the primary contributor to systemic alterations in adiponectin, IL6, and TNF α , at least in canine obesity. Further investigation is required to determine whether visceral obesity in dogs poses increased risk for metabolic disorders, such as insulin resistance, hepatic steatosis, and atherosclerosis, similar to findings in human subjects.

Table 16. A summary of associations between adipose tissue anatomic location and adipokines concentrations in medium.

	Dogs	Cats
Adiponectin	M < SC	N
Interleukin -6	M > SC	M > SC
Tumor Necrosis Factor - α	M > SC	N

M: mesenteric; SC: subcutaneous; N: no significant association

1.3.6. Effect of troglitazone on adipokines

Troglitazone treatment had significant effects on medium concentrations of adiponectin, IL6, and TNF α both in mesenteric and subcutaneous canine adipose tissue (Study 1.2.4). Mean adiponectin concentrations were 100% and 39% higher, mean IL6 concentrations were 14% and 44% lower, and mean TNF α concentrations were 19% and 37% lower in troglitazone-treated compared to control cultures in mesenteric and subcutaneous cultures, respectively. No significant effect of troglitazone on secretion of adiponectin was revealed in feline cultures (Study 1.2.5). Potential effects of troglitazone on secretion of IL6 and TNF α were not evaluated in this study due to unavailability of samples from cats (Table 17).

Adiponectin

PPAR γ is expressed predominantly in the adipose tissue and its expression is decreased in obese dogs (Gayet et al., 2007) and cats (Hoenig et al., 2008). The stimulatory effect of troglitazone on adiponectin secretion demonstrated in dogs is in agreement with studies on primary adipocytes culture from humans and rodents and adipocytes cell lines (Motoshima et al., 2002; Bodles et al., 2006; Lorente-Cebrian et al., 2006; Phillips et al., 2008; Tishinsky et al., 2011), and consistent with finding in dogs as well, including the reported PPAR γ mRNA expression in canine adipose tissue, the findings of increased circulating concentrations of adiponectin following oral administration of pioglitazone in Beagle dogs (Kasai et al., 2008), and the reported increased expression of adiponectin in canine adipocytes differentiated in culture in response to rosiglitazone (Ryan et al., 2010).

Although PPAR γ -agonists have generally been shown to increase adiponectin mRNA expression and protein secretion in human and rodent adipocytes (Motoshima et al., 2002; Bodles et al., 2006; Lorente-Cebrian et al., 2006; Tishinsky et al., 2011), the effect was found to be both depot- and time-dependent. In one study in isolated human adipocytes, a stimulatory effect was demonstrated in visceral but not subcutaneous tissue (Motoshima et al., 2002), but in another study in humans adipose tissue explants, a stimulatory effect was present in subcutaneous and not visceral tissue (Phillips et al., 2008). Furthermore, an effect of PPAR γ agonist to increase adiponectin secretion was present from days 2-6 of culture while no effect was seen during the first 2 days (Motoshima et al., 2002; Phillips et al., 2008). In the present studies in dogs and cats, secretion of adiponectin was evaluated in the first 2 days of culture only; therefore potential effects of troglitazone following additional days in feline culture may be possible. Although a response to PPAR γ agonist cannot serve to attest to the viability of feline adipocytes culture in this study, the presence of detectable adiponectin protein and the significant positive association of its concentration to PAV strongly support the validity of this culture system.

Interleukin-6 and Tumor Necrosis Factor- α

The inhibitory effect of troglitazone on IL6 and TNF α secretion in canine adipose tissue culture is in agreement with studies in human mononuclear cells (Jiang et al., 1998; Skurk et al., 2006) and consistent with the recently reported decreased expression of IL6 and TNF α in canine adipocytes differentiated in culture in response to rosiglitazone (Ryan et al., 2010). However, in contrast to the findings in the present study, no change in IL6 and TNF α protein secretion was

documented in this previous study. The disagreement in the findings regarding the cytokine protein secretion between these two studies may be explained by the use of differentiated adipocytes with a lower baseline secretion of inflammatory cytokines in the previous study compared to the SVCs used in the present study, as well as a lower dose of the PPAR γ agonist used in the previous study. In addition, the discrepancy between gene expression and protein secretion in the previous study may reflect time course differences between expression and the release of the encoded protein.

The effect of troglitazone on the secretion of adipokines in canine adipose tissue culture suggests that obesity-related alterations in circulating adipokines concentrations may be attenuated by a PPAR γ agonist treatment. Further investigation is required to determine the role of PPAR γ agonist in therapy for diseases and metabolic disorders associated with obesity. Although darglitazone treatment was shown to improve insulin sensitivity and lipid metabolism in obese cats (Hoenig and Ferguson, 2003), the study reported here does not provide evidence to indicate that this therapeutic effect is mediated through stimulation of adiponectin secretion from adipocytes.

Table 17. A summary of associations between troglitazone treatment and adipokines concentrations in medium.

	Dogs	Cats
Adiponectin	↑	N
Interleukin -6	↓	-
Tumor Necrosis Factor - α	↓	-

↑: significant positive association; ↓: significant negative association; N: no significant association; -: data unavailable

1.3.7. *Effect of n3PUFAs on adipokines*

Significant associations were demonstrated between circulating concentrations of n3PUFAs and adipokines in dogs (Study 1.2.1) and cats (Study 1.2.3). DPA was the single n3PUFA associated with concentrations of adiponectin, leptin, and IL6 in dogs, whereas associations of adiponectin and leptin were present primarily with EPA in cats, suggesting these FAs are the best indicators of the effect of n3PUFAs on adipokines. The effects of n3PUFAs on adipokines were different between obese and non-obese cats, but were similar in dogs of variable body condition. A positive association of n3PUFA with adiponectin was found in dogs and obese cats and a negative association was found in non-obese cats; a positive association of n3PUFA with leptin was revealed in dogs and non-obese cats; and a negative association of n3PUFA with IL6 was found in dogs (Table 18).

Since these findings suggested a regulatory role of long chain n3PUFAs on adiponectin and leptin production and secretion in dog and cats, a longitudinal study to determine the effects of dietary supplementation with n3PUFAs was conducted in dogs (Study 1.2.2). Fish oil (rich in EPA and DHA) was administered at a daily dose of 220 mg/kg for 30 days; this dose is within the range recommended for the treatment of hyperlipidemia in dogs (Schenck, 2006; Xenoulis and Steiner, 2010). This supplementation regimen increased circulating concentrations of adiponectin but did not result in a measurable difference in circulating concentrations of leptin.

An effect of long chain n3PUFAs to increase circulating concentrations of adiponectin may be mediated through a direct stimulatory effect on the adipocytes to secrete adiponectin or through their anti-inflammatory effects, indirectly leading to decreased inhibition on adiponectin secretion.

To evaluate the direct effect of n3PUFAs on secretion of adipokines from adipose tissue, concentrations of adipokines secreted in culture were determined in response to treatment with EPA (Studies 1.2.4 and 1.2.5). Mature adipocytes and SVCs were studied separately to allow differentiation of the effect of FAs on the secretion of adipokines from these subfractions of adipose tissue, specifically, adiponectin and leptin from mature adipocytes and IL6 and TNF α from SVCs. The range of concentrations studied (25-100 μ M) was chosen to include the typical range at which n3PUFA circulate in vivo (Spiegelman, 1998). n6PUFAs and SFAs normally circulate in much higher concentrations and elevations of n3PUFA concentrations by dietary supplementation results in partial substitution of these abundant FAs by n3PUFAs. Therefore, the effect of EPA was compared to AA, an n6PUFA, and PA, a SFA. Direct effects of FAs were revealed on SVCs in canine and feline adipose tissue cultures and on mature adipocytes in canine but not feline cultures. Specifically, stimulation of IL6 secretion from SVCs by PA in dogs and AA in cats, and inhibition of IL6 by EPA in dogs, as well as stimulation of adiponectin secretion from mature adipocytes by EPA and inhibition by PA in dogs.

Fatty Acids

The findings of associations between circulating concentrations of adipokines and DPA alone in dogs, rather than other long chain n3PUFAs (EPA and DHA) or n3PUFA of shorter chain length (ALA) disagree with a previous study on lipid metabolism in mice that demonstrated intermediate effects of DPA between those of EPA and DHA and concluded that the beneficial effects of DPA are not superior to those of EPA or DHA (Gotoh et al., 2009). However, DPA is the main metabolite of ALA in cell membranes and therefore it may serve as

an important circulating or tissue reservoir for either EPA or DHA synthesis. In a dietary ALA-supplementation study in dogs (Dunbar et al., 2010), enrichment with DPA was demonstrated in the phospholipid and triglyceride fractions, but not the cholesteryl-ester fraction, indicating tissue synthesis and conservation of DPA, which then may serve as a substrate for DHA synthesis in the tissues. Therefore, the associations revealed in this study with DPA may be indicative of an effect of long chain n3PUFAs in general. In addition, if the potential biologic effect of n3PUFAs is carried out by the long chain n3PUFAs, then the lack of effect of the shorter chain n3PUFA might be explained by the limited conversion of ALA to the longer chain derivatives (EPA and DHA) due to low Δ -6 desaturase activity, especially in cats (Rivers et al., 1975).

Following fish oil supplementation to dogs, serum concentrations of total n3PUFAs increased as expected. Among the individual n3PUFAs, EPA and DHA concentrations showed the largest increases, but increases were detected in concentrations of DPA and ALA as well, and may indicate post absorption metabolism of the ingested EPA and/or DHA. A decrease in serum concentrations of n6PUFA was demonstrated post supplementation and most likely represents the decrease in their proportion of total FA concentrations, but may also result from an inhibiting effect of ALA on the conversion of LA to AA (Hall et al., 2006).

Adiponectin

A positive association between serum concentrations of n3PUFAs and adiponectin was revealed in dogs and in obese cats, whereas a negative association was found in non-obese cats. Concentration of DPA explained 21% of the variability in adiponectin in dogs; concentrations of

EPA and DHA each accounted for 22% of the variability in adiponectin in the obese cats; and concentration of EPA explained 18% of the variability in adiponectin in non-obese cats. The findings in dogs and obese cats are in agreement with the reported direct relationship between circulating plasma adiponectin and n3PUFA concentrations in human subjects (Fernandez-Real et al., 2005; Hernandez-Morante et al., 2007; Kim et al., 2010).

The finding of increased concentrations of adiponectin following supplementation with fish oil coupled with no effect on body condition further supports the presence of a regulatory role of n3PUFAs on adiponectin production and secretion in healthy dogs. These results are in agreement with previous reports of increased circulating concentrations of adiponectin following prescription EPA treatment in obese human subjects (Itoh et al., 2007) and a dietary intervention to decrease n6/n3 FA ratio in non-obese healthy human subjects (Guebre-Egziabher et al., 2008), as well as multiple studies in rodent models of obesity (Rossi et al., 2005; Flachs et al., 2006; Itoh et al., 2007; Perez-Matute et al., 2007) and non-obese rodents (Neschen et al., 2006; Duda et al., 2007; Higuchi et al., 2008). A lack of effect of n3PUFA on adiponectin concentrations was reported in human subjects in two other studies (Kratz et al., 2008; Tsitouras et al., 2008) and may be explained by a smaller sample size and/or different study design.

A direct effect of n3PUFA to increase adiponectin secretion from adipocytes was demonstrated in dogs, but not in cats. EPA and PA had opposing effects on adiponectin secretion from mature canine adipocytes. At a lower dose, PA had an inhibitory effect on secretion of adiponectin and this secretion was higher in response to EPA than PA both in mesenteric and subcutaneous adipose tissue. At a higher dose, EPA had a stimulatory effect on adiponectin secretion in subcutaneous depot only. In contrast, secretion of adiponectin from mature feline adipocytes was not altered by any of the FAs studied.

The effect of EPA to increase adiponectin secretion from primary mature canine adipocytes is in agreement with recent reports of increased adiponectin secretion from cultured human adipocytes (Tishinsky et al., 2011) as well as mouse adipocytes cell line- 3T3-L1 (Oster et al., 2010) in response to EPA or DHA treatment. However, previous studies on the effect of EPA treatment on adiponectin gene expression and protein secretion reported contradictory results. A decrease in adiponectin gene expression and protein secretion was initially demonstrated in primary rat adipocytes (Lorente-Cebrian et al., 2006). Subsequent studies in mouse adipocytes cell line (3T3-L1) reported either no change in adiponectin gene expression and protein secretion (Itoh et al., 2007) or a decrease in adiponectin gene expression (Bueno et al., 2008).

The effect of EPA could potentially be mediated through upregulation and activation of PPAR γ . This potential mechanism is supported by the stimulatory effect of troglitazone on adiponectin secretion demonstrated in canine adipocytes, while the lack of effect of EPA on adiponectin secretion from mature feline adipocytes is in line with the failure of troglitazone to alter adiponectin secretion.

PUFA were shown to interact directly with PPAR γ *in vitro* in concentrations consistent with those found in human serum and were therefore suggested to function as natural ligands for this receptor (Kliwer et al., 1997). More specifically, DPA and DHA and their metabolites have been shown to act as PPAR γ agonists (Yamamoto et al., 2005; Groeger et al., 2010). Moreover, EPA was demonstrated to increase PPAR γ gene expression in primary human adipocytes (Chambrier et al., 2002). Other studies report contradictory results, a significant decrease in PPAR γ gene expression was reported in primary rat adipocytes (Lorente-Cebrian et al., 2006), and no change was found in mouse adipocytes cell line (3T3-L1) (Oster et al., 2010). Supporting

the notion that EPA increases adiponectin secretion at least partially through a PPAR γ -mediated mechanism is the finding of attenuation of the effect of EPA by a PPAR γ antagonist in human adipocytes (Tishinsky et al., 2011) as well as the inhibition of increase in adiponectin concentrations with a PPAR γ inhibitor in mice fed a high n3PUFA diet (Neschen et al., 2006)

The apparently conflicting reports regarding the effect of EPA on adiponectin may be related to species or study design differences including the range of EPA dose used and the outcome determined, specifically gene expression, cellular protein expression, or protein secretion. The effect of EPA on adiponectin gene expression and protein secretion was consistently positive in human adipocytes (Tishinsky et al., 2011) and negative in rat adipocytes (Lorente-Cebrian et al., 2006). The effect on 3T3-L1 adipocytes, on the other hand, was variable, with a positive effect documented with a lower dose (125 μ M) of EPA (Oster et al., 2010) and either no effect (Itoh et al., 2007) or a decrease (Bueno et al., 2008) with a higher dose range (200-250 μ M). A dose dependent effect of EPA on adiponectin may be related to the documented dose dependent effect of this FA on PPAR γ expression, with a maximal effect between 25-50 μ M and a progressive decline at higher concentrations (Chambrier et al., 2002). In accordance with these findings, a decrease in PPAR γ expression was reported in rat adipocytes with EPA treatment at a range of 100-200 μ M (Lorente-Cebrian et al., 2006). Alternatively, the negative results with the higher doses of EPA may result from increased lipid peroxidation leading to oxidative stress, which had been shown to decrease adiponectin expression (Soares et al., 2005). Consistent with these previous studies, a positive effect of EPA on adiponectin secretion was demonstrated using a 50 μ M dose in the present study. In addition, these results suggest that canine adipocytes are more closely related to human rather than rat or mouse adipocytes. Indeed, a stimulatory effect of EPA on adipocytes was reported in

subcutaneous tissue from human subjects (Tishinsky et al., 2011). Other studies in human adipocytes demonstrated an effect of PPAR γ agonist to increase adiponectin secretion from days 2-6 of culture while no effect was seen during the first 2 days (Motoshima et al., 2002; Phillips et al., 2008). A similar time-dependent pattern of adiponectin secretion in cats would provide a potential explanation of the lack of response to troglitazone as well as EPA, as secretion of adiponectin was evaluated in the first 2 days of culture only.

The finding of an effect in subcutaneous but not mesenteric tissue in dogs suggests that at this dose EPA was able to activate PPAR γ but not upregulate its expression, so that a clear effect was present in the subcutaneous tissue where the basal expression of PPAR γ is higher rather the mesenteric tissue with a lower basal expression.

N3PUFAs may also have an effect to increase adiponectin secretion through substitution of SFAs, such as PA that was demonstrated to decrease adiponectin secretion in canine adipocytes. The inhibitory effect of PA on adiponectin secretion from mature canine adipocytes is in agreement with previous reports on 3T3-L1 cell line (Bueno et al., 2008; Chakrabarti et al., 2009). However, other studies report conflicting results. No effect of PA on adiponectin secretion or cellular content was revealed in primary human adipocyte culture (Tishinsky et al., 2011), and PA also failed to affect secretion of adiponectin in two additional studies in 3T3-L1 (Oster et al., 2010; Szkudelski et al., 2011), but increased cellular adiponectin in the former. Yet another study on 3T3-L1 demonstrated an increase in adiponectin secretion and cell content in response to PA (Takahashi et al., 2008).

An explanation for the conflicting reports regarding the effect of PA on secretion of adiponectin is not clearly apparent. A potential mechanism for an inhibitory effect of PA on adiponectin secretion has been demonstrated in 3T3-L1 culture (Chakrabarti et al., 2009), where

PA augmented expression of 12/15-lipoxygenase and treatment of adipocytes with products of this enzyme resulted in decreased expression of adiponectin. Additionally, a potential inhibitory effect of PA on secretion of adiponectin may be mediated through its effect to increase secretion of IL6 from adipocytes (Ajuwon and Spurlock, 2005; Chakrabarti et al., 2009), since IL6 had been shown to down regulate adiponectin expression (Bruun et al., 2003).

Leptin

A positive association between serum concentrations of an n3PUFA and leptin was revealed in dogs and in non-obese cats. Concentration of DPA explained 8% of the variability in leptin in dogs and concentration of EPA accounted for 23% of the variability in leptin in the non-obese cats. These findings are in contrast to the findings of increased leptin concentrations associated with decreased n3PUFA concentrations in human patients with acute myocardial infarction (Oda et al., 2005). The fish-oil supplementation study in dogs failed to support the presence of a regulatory effect of n3PUFAs on leptin production. The presence of a direct effect of n3PUFA on leptin secretion from adipocytes could not be evaluated due to the unavailability of a sensitive assay to detect leptin concentrations secreted to the medium.

The positive association between circulating concentrations of DPA and leptin in dogs suggests that DPA may up-regulate leptin production in dogs. However, dietary fish oil supplementation failed to increase leptin concentrations. The differential effect of EPA on leptin concentrations in cats, suggests that EPA may up-regulate leptin production in non-obese, but not in obese cats. Previous studies in humans and rodent models report conflicting results regarding the effect of n3PUFA on leptin. Some studies report an increase (Peyron-Caso et al.,

2002; Hynes et al., 2003; Rossi et al., 2005), some a decrease (Hun et al., 1999; Wang et al., 2002), while others revealed no change (Lara et al., 2007; Wang et al., 2008) in leptin concentrations or mRNA expression following fish oil supplementation. The reason for these variable findings is unclear but could be related to study design, including differences in dose regimen and inclusion of subjects of variable body condition. In addition, species differences in response of leptin expression to PPAR γ agonists may have led to variable results in these studies. Increased leptin expression has been recently reported in cultured canine adipocytes in response to Rosiglitazone (Ryan et al., 2010) and supports the possibility of PPAR γ activation by DPA in dogs or EPA in non-obese cats. In contrast, an inhibitory effect of PPAR γ agonists on leptin expression is reported in humans and rodents (De Vos et al., 1996; Kallen and Lazar, 1996; Lee and Fried, 2009).

Interleukin-6

A negative association was demonstrated between circulating concentrations of DPA and IL6 in healthy dogs and is in agreement with the anti-inflammatory effect of n3PUFA (Suganami et al., 2005; Todoric et al., 2006; Groeger et al., 2010). These results are consistent with the reported decrease in serum IL6 activity in dogs fed an n3PUFA-rich diet (LeBlanc et al., 2008).

Different patterns of IL6 secretion in response to FAs were demonstrated in canine and feline SVCs cultures. A differential dose dependent effect of EPA and PA was demonstrated in canine subcutaneous adipose tissue SVCs. Secretion of IL6 was decreased by EPA treatment and increased by PA treatment. In the mesenteric depot, EPA treatment tended to decrease while PA treatment did not change IL6 secretion. In addition, intermediate and high doses of FA had an

effect on subcutaneous tissue while only the highest dose yielded an effect on mesenteric tissue. In feline cultures, AA had a stimulatory effect on IL6 secretion from adipose tissue SVC both in mesenteric and in subcutaneous depots, while EPA and PA did not change IL6 secretion.

The stimulatory effect of PA and inhibitory effect of EPA on secretion of IL6 from canine SVCs are in general agreement with studies utilizing LPS-stimulated macrophage cell lines. In murine macrophages (J774), secretion of IL6 was increased by PA, decreased by DHA, and did not change by EPA (Martins de Lima-Salgado et al., 2011); in human macrophages (THP-1), it was not affected by PA but was decreased by various PUFA (Zhao et al., 2005; Wang et al., 2009). These studies all demonstrated higher secretion of IL6 by PA compared to EPA or other PUFA, similar to the findings in the present study, where PA resulted in higher IL6 secretion than EPA. In addition to its effect on macrophages, PA has also been shown to induce secretion of IL6 from murine adipocytes (Chakrabarti et al., 2009) and adipocytes cell line (Ajuwon and Spurlock, 2005) as well as human endothelial cells (Staiger et al., 2004b). It is possible that secretion of IL6 from preadipocytes and endothelial cells in response to PA contributed to the increased overall secretion of IL6 from the stromovascular fraction in this study as well.

The differential effect of PA and EPA on IL6 secretion in canine SVCs may be mediated through their opposite interactions with macrophage toll-like receptor 4 and subsequent induction of NF κ B, which plays a central role in modulating the expression of immunoregulatory genes including transcription of cytokines. PA has been shown to activate this receptor while PUFA suppress its signaling pathway (Lee et al., 2001; Lee et al., 2003; Ajuwon and Spurlock, 2005). Moreover, the inhibitory effect of EPA on IL6 secretion may be mediated through its PPAR γ ligand properties, as activation of PPAR γ in macrophages and endothelial cells has been

shown to inhibit the expression of proinflammatory cytokines, including IL6, by inhibiting Nuclear Factor- κ B signaling (Jiang et al., 1998). The different pattern of effect of EPA and PA between the mesenteric and subcutaneous tissues may be due to the higher basal IL6 secretion in the mesenteric depot, so that PA did not further stimulate its secretion and a higher concentration of EPA was required to attenuate its secretion.

This unique pattern of IL6 response to different FA in feline adipose tissue SVC may represent a peculiarity of this species, but can also be viewed as generally consistent with the role of AA in enhancing immune function (Zheng et al., 1999). In line with these findings in cats, a previous study using an in vitro model of intestinal inflammation demonstrated increased activation of the transcription factor Nuclear Factor- κ B by AA compared to EPA (Ramakers et al., 2007). Nuclear Factor- κ B is a key regulator of the inflammatory response, including transcription of IL6 among other cytokines. AA may potentially induce Nuclear Factor- κ B activation in feline SVC, leading to the demonstrated increase in secretion of IL6 in the present study.

Tumor Necrosis Factor- α

A positive association was revealed between circulating concentrations of TNF α and PA in healthy dogs and is in agreement with the suggested pro-inflammatory effect of SFAs (Suganami et al., 2005; Todoric et al., 2006; Groeger et al., 2010).

Surprisingly, treatment of canine SVCs with all FAs resulted in decreased secretion of TNF α . The reduction of TNF α secretion was present both in mesenteric and subcutaneous tissues at lower FA doses, but only in the subcutaneous depot at the highest dose. The effect of FA on

secretion of TNF α from feline SVC was less consistent, and included increased secretion in response to EPA treatment and decrease secretion in response to AA and PA treatment.

These results are surprising in view of the findings for IL6 secretion both in dog and in cats and are in contrast to previous studies that implicated SFA from hypertrophied adipocytes in induction of TNF α in macrophages, while EPA suppressed this induction (Itoh et al., 2007). Other in vitro studies utilizing murine (J774) (Martins de Lima-Salgado et al., 2011) or human (THP-1) (Wang et al., 2009) macrophage cell lines demonstrated no change in TNF α secretion with PA treatment but reduction with variable PUFA. In murine adipocytes cell line (3T3-L1), PA increased and DHA did not change TNF α mRNA expression and protein secretion (Bradley et al., 2008). These previous studies all showed higher TNF α secretion with PA compared to PUFAs. A similar pattern was present in canine SVCs culture in the present study with treatment of 50 μ M in the subcutaneous depot, with AA and EPA treatments associated with decreased secretion while there was no difference with PA treatment. However, this pattern was not consistent over different doses and across tissue depots.

An explanation for the undifferential effect of FAs to decrease TNF α secretion in canine SVC culture is not clearly apparent. The possibility that degradation of this cytokine in the culture media occurred prior to media collection cannot be entirely ruled out, but could not explain the difference between the measured concentrations of the control and FA-treated cultures. It is possible that differential effects of FA on mRNA expression of TNF α were present, but were not reflected in concentration of the secreted protein as was previously reported for the effect of PA on murine adipocytes (Ajuwon and Spurlock, 2005). Such discordance between levels of mRNA expression and measured protein concentration may occur due to suppressed

cleavage of transmembrane TNF α , as was reported to occur in differentiated adipocytes (Xu et al., 2002).

The stimulatory effect of EPA on TNF α secretion from feline SVC is surprising in view of the known anti-inflammatory effect of this FA. Moreover, an explanation for the different effect of EPA and AA on secretion of TNF α compared to IL6 is not clearly apparent. Interestingly, a similar pattern of response of IL6 and TNF α to another anti-inflammatory agent was present in differentiated canine adipocytes, where mRNA expression of TNF α was increased while IL6 expression was decreased in response to dexamethasone (Ryan et al., 2010). In addition, an opposite effect EPA and AA has been demonstrated in an in vitro model of intestinal inflammation where EPA increased IL6 secretion and AA increased TNF α secretion (Ramakers et al., 2007). IL6 was previously shown to suppress TNF α production in skeletal muscle (Pedersen et al., 2003). Although the presence of a similar interaction between these cytokines has not been shown in adipose tissue or macrophages, it may be speculated that IL6 secreted into the culture medium had an inhibitory effect on the release of TNF α in the present study. Such an interaction between secretion of IL6 and TNF α may provide an explanation for the opposite effect of these FA as well as the apparently stimulatory effect of EPA on TNF α secretion.

Differential effect of n3PUFAs with body condition

In the longitudinal study, dogs that responded to fish oil supplementation with increased circulating adiponectin concentrations had significantly lower BCS compared to dogs that experienced a decrease in adiponectin concentration. This finding may suggest that the supplementation regimen in this study was not able to up-regulate PPAR γ expression in the

adipose tissue of dogs with higher BCSs, but was successful in activating the existing PPAR γ in the adipose tissue of leaner dogs. In contrast, the significant positive association between the n3PUFAs, EPA and DHA, and concentrations of adiponectin in obese but the negative association between EPA and adiponectin in non-obese cats suggest that while n3PUFA concentrations in un-supplemented cats may up-regulate PPAR γ expression in adipose tissue of obese cats, it is incapable of activating PPAR γ present in adipose tissue of leaner cats. Moreover, EPA had a negative effect on concentrations of adiponectin in non-obese cats. A potential explanation for this finding is not clearly apparent. Some clarification may be provided by the reported dose dependent effect of EPA on PPAR γ expression in isolated human adipocyte, showing an initial increase and a later decrease of the expression at high EPA concentrations (Chambrier et al., 2002). Higher EPA concentrations could lead to suppression of the already high baseline PPAR γ expression in relatively lean cats, resulting in lower circulating adiponectin concentrations.

The finding of a positive effect of n3PUFA on adiponectin concentrations in obese but not in non-obese cats may also suggest that this effect is mediated through anti-inflammatory effects of n3PUFA, since a larger effect is expected in obese cats due to a more severe inflammatory process in the adipose tissue compared to non-obese. For the same reason, the finding of lower BCS in the dogs that responded to fish oil supplementation with increased adiponectin concentrations does not provide support for anti-inflammatory effect as the mechanism by which n3PUFA exerts their effect. It is possible that adipose tissue inflammation was not present to a significant extent in these dogs as all dogs were relatively lean (BCS:4-6). Therefore, a potential effect of fish oil to increase adiponectin through an anti-inflammatory effect may be possible in dogs with higher BCS.

Another potential explanation for a differential response to fish oil supplementation with the majority of dogs showing increases in adiponectin concentrations may be the presence of gene-diet interaction, similar to findings in humans. A relatively common polymorphism in the PPAR γ gene in humans results in an opposite response to agonist as exemplified by an increased risk for myocardial infarct with higher PUFA intake in carriers of these polymorphism as opposed to the decreased risk in non-carriers (Stumvoll and Haring, 2002; Ruiz-Narvaez et al., 2007).

The presence of a significant positive association between EPA and leptin in non-obese but not in obese cats may be related to the smaller number of obese compared to non-obese cats in this study. Alternatively, EPA may up-regulate leptin production in non-obese but not in obese cats due to the high leptin concentrations resulting from the presence of leptin resistance in obese cats.

Table 18. A summary of associations between n3PUFAs and adipokines concentrations in serum (A), effect of dietary fish oil supplementation on serum concentrations (B), and effect of FAs treatment on medium concentration (C).

A.

	Dogs	Obese cats	Non-obese cats
Adiponectin	↑↑	↑	↓
Leptin	↑	N	↑
Interleukin -6	↓	-	-
Tumor Necrosis Factor - α	N	-	-

Table 18 (cont'd)

B.

	Dogs
Adiponectin	↑
Leptin	N

C.

	Dogs	Cats
Adiponectin	EPA > Control > PA	N
Interleukin -6	EPA < Control < PA	AA > Control
Tumor Necrosis Factor - α	All FAs < Control	EPA > Control > AA/PA

↑: significant positive association/increase; ↓: significant negative association; N: no significant association/change; -: data unavailable

1.3.8. Effect of n3PUFAs on insulin and triglyceride

N3PUFAs were associated with serum concentrations of triglyceride both in dogs and in cats and with circulating concentrations of insulin in cats only (Studies 1.2.1 and 1.2.3). These associations were present with EPA alone in cats and DPA and ALA in dogs; and were different in obese and non-obese cats, but similar in dogs of different body condition. Fish oil supplementation for 30 days failed to alter concentrations of triglyceride or insulin in dogs (Study 1.2.2).

Insulin

An inverse relationship between serum concentrations of insulin and EPA was found in obese, but not in the non-obese cats. Concentration of EPA explained 25% of the variability in insulin in these cats. No association between any of the n3PUFAs and concentrations of insulin was revealed in dogs and no difference was detected following dietary supplementation with fish oil.

The inverse relationship between serum concentrations of insulin and EPA found in obese cats suggest a potential regulatory effect of EPA on insulin sensitivity, and consequently insulin concentrations in obese cats. These findings agree with the beneficial effects of fish oil consumption on insulin sensitivity in human (Lombardo and Chicco, 2006) and the decreased insulin concentrations in obese cats following feeding a diet rich in n3PUFAs compared to a diet high in SFA (Wilkins et al., 2004).

The lack of a significant association between concentrations of any of the n3PUFAs and insulin in dogs as well as the finding of no significant effect of dietary supplementation with fish oil do not provide support for a regulatory role of n3PUFAs on insulin sensitivity. These findings are in general agreement with an earlier study that revealed no change in insulin-sensitivity in 6 healthy dogs following 6 months of fish oil supplementation (Irvine et al., 2002). However, the dog with the highest concentrations of insulin in that study showed improved insulin sensitivity following supplementation. Therefore, it is possible that n3PUFAs may have a beneficial effect on insulin sensitivity once insulin resistance is present, but do not further improve already normal measures, as was the case in the dogs in the present supplementation study. Furthermore, the results of these studies cannot exclude the possibility for a potential effect of n3PUFAs on insulin sensitivity in dogs since baseline insulin concentrations are considered a relatively insensitive indicator of insulin resistance. In addition, a different dose or duration of supplementation may be required for an effect on insulin sensitivity.

A possible effect of n3PUFAs on insulin sensitivity may be mediated by increased adiponectin production and secretion, as adiponectin has profound insulin-sensitizing properties. Nevertheless, n3PUFAs may have additional insulin-sensitizing effects that are not directly related to increased adiponectin: up-regulation and activation of PPAR γ by n3PUFAs in adipose tissue lead to transformation to small adipocytes that are more sensitive to insulin; increased cell membrane incorporation of n3PUFAs in insulin target tissues results in improved insulin activity; n3PUFAs may stimulate FA oxidation in the liver and skeletal muscle, decreasing tissue lipid accumulation and improving insulin action; n3PUFAs may also improve glucose utilization by attenuation of obesity-induced GLUT4 down-regulation in insulin target tissues (Peyron-Caso et al., 2002; Ukropec et al., 2003; Lombardo and Chicco, 2006). The effect of n3PUFAs to

improve insulin sensitivity may also be mediated through reduction of adipose tissue TNF α concentrations, as TNF α impairs insulin signaling by inhibition of the tyrosine kinase activity of the insulin receptor (Hotamisligil, 1999).

Triglyceride

An inverse relationship between serum concentrations of triglyceride and EPA was found in obese, but not in non-obese cats (Study 1.2.3). Concentration of EPA explained 32% of the variability in triglyceride in these cats. Both DPA and ALA were associated with concentrations of triglyceride concentrations in dogs (Study 1.2.1); however, these associations were opposite, rendering the potential effect of n3PUFA on triglyceride concentration in dogs speculative. DPA was inversely related while ALA was positively related to triglyceride concentrations, each accounting for 14% of the variability of its concentrations. No difference in triglyceride concentrations was detected in dogs following dietary supplementation with fish oil.

The findings of an inverse relationship between concentrations of n3PUFAs and triglyceride both in dogs and cats are in agreement with previous reports in dogs (LeBlanc et al., 2005) and humans (Lombardo and Chicco, 2006; McKenney and Sica, 2007). However, early studies using dogs as models for human disease as well as studies in healthy cats revealed no effect of fish oil treatment on concentrations of triglyceride (Harris, 1997; Plantinga and Beynen, 2003; Wilkins et al., 2004). The lack of effect of fish oil on triglyceride concentrations may be related to the relatively short supplementation period and the fact that all dogs were healthy without marked hyperlipidemia.

The opposite effect of ALA and DPA on triglyceride concentrations may not be simply explained, but it is possible that low ALA concentration is a marker for higher DPA concentrations due to the conversion of ALA to DPA.

The hypolipidemic effect of n3PUFAs is due to the suppression of transcription of genes encoding lipogenic enzymes and increased FA oxidation in the liver (Lombardo et al., 1996). Additional beneficial effects on hypertriglyceridemia include stimulation of lipoprotein lipase activity and decrease in intestinal absorption of lipid and glucose (Levy et al., 1993). The effect of n3PUFAs to decrease triglyceride concentrations may also be mediated through suppression of TNF α , which stimulates VLDL secretion from the liver and increases lipolysis in adipose tissue (Feingold and Grunfeld, 1992).

Table 19. A summary of associations between n3PUFAs and concentrations of insulin and triglyceride (A) and effect of dietary fish oil supplementation (B).

A.

	Dogs	Obese cats	Non-obese cats
Insulin	N	↓	N
Triglyceride	↓	↓↓	N

B.

	Dogs
Insulin	N
Triglyceride	N

↓: significant negative association; N: no significant association/change.

1.3.9. Summary

N3PUFAs were demonstrated as a determining factor on adipokines secretion and concentrations in healthy dogs and cats. These effects were differential between obese and non-obese cats but were similar for dogs of variable BCSs.

In healthy dogs, DPA was directly related to concentrations of adiponectin and leptin, and dietary supplementation with fish oil increased concentrations of adiponectin but did not alter concentrations of leptin. In obese cats, concentrations of EPA and DHA were directly related to adiponectin concentrations, while in non-obese cats EPA concentrations were inversely related to concentrations of adiponectin and directly related to concentrations of leptin.

The effects of n3PUFA on circulating concentrations of adiponectin may be explained by the local effects of n3PUFAs as opposed to other FAs (n6PUFAs or SFAs) on secretion of adiponectin as well as inflammatory cytokines from adipose tissue.

In canine adipose tissue culture, EPA was demonstrated to have a stimulatory effect on adiponectin secretion from mature adipocytes and an inhibitory effect on IL6 secretion from SVCs. In contrast, PA had an inhibitory effect on adiponectin secretion from mature adipocytes and a stimulatory effect on IL6 secretion from SVCs. Therefore, the effect of EPA to increase adiponectin secretion from adipocytes and decrease IL6 secretion from SVCs may be conveyed either directly or by substitution for SFA as PA, which has opposite effects on adiponectin secretion from adipocytes and IL6 secretion from SVCs. The effect of EPA to decrease IL6 may also contribute to the increase in adiponectin secretion, as IL6 may have an inhibitory effect on adiponectin secretion.

Although associations between serum concentrations of adipokines and n3PUFAs were revealed with DPA alone, EPA was shown to have a direct effect on mature adipocytes and SVCs in culture, suggesting that conversion between these FAs occurs and that DPA may be an indicator of long chain n3PUFAs concentrations.

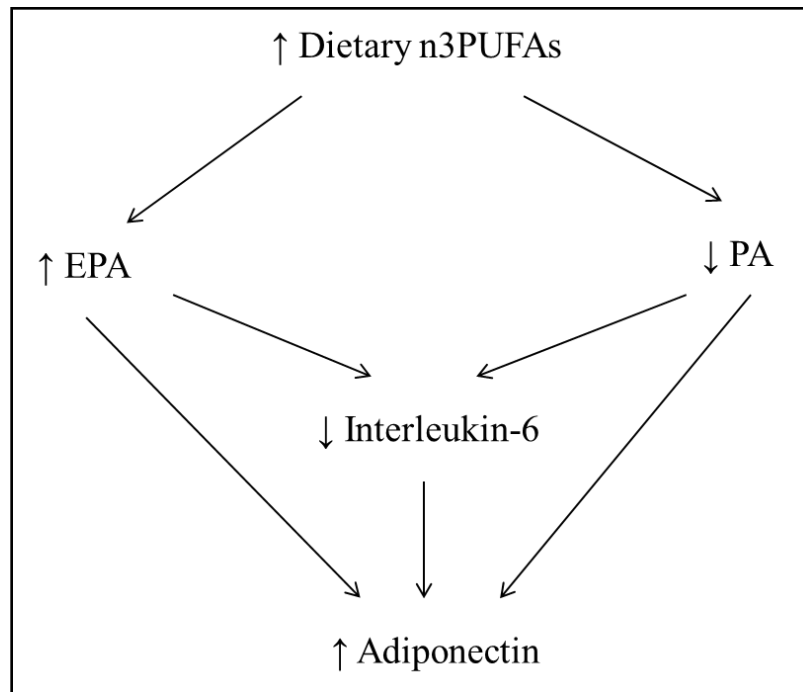
In feline adipose tissue culture, AA was demonstrated to have a stimulatory effect on secretion of IL6 from SVCs, but findings of the present study did provide support for the presence of a direct effect of FAs on secretion of adiponectin from mature adipocytes. Nevertheless, an increase of IL6 secretion by AA may lead to a decrease in adiponectin secretion, and therefore substitution of AA by EPA would potentially result in an increase in secretion of adiponectin.

N3PUFAs were related to concentrations of triglyceride. DPA in dogs and EPA in obese cats were inversely related to concentrations of triglyceride, supporting a hypolipidemic effect of these FAs in dogs and cats. EPA was also inversely related to insulin concentrations in obese cats, supporting an insulin-sensitizing effect. These findings suggest beneficial effects of n3PUFA on hyperlipidemia in dogs and cats, and insulin-resistance in cats.

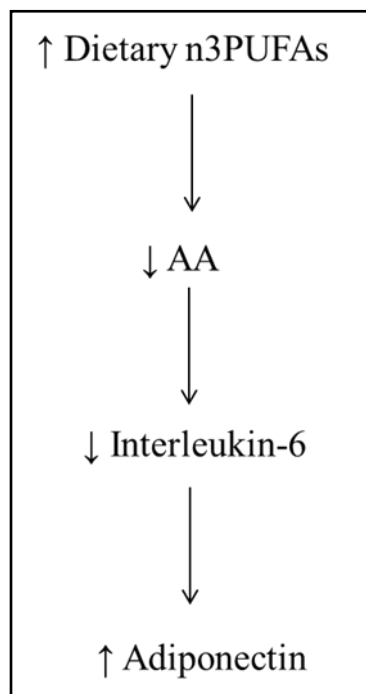
Figure 11. Proposed mechanisms for the potential effects of n3PUFAs to increase adiponectin concentrations in dogs (A) and cats (B).

Figure 11 (cont'd)

A. Dogs



B. Cats



CHAPTER 2

ADIPOKINES AND N3PUFAS IN OBESITY-RELATED DISEASES IN DOGS AND CATS

2.1. Introduction

2.1.1. Obesity-related diseases, adipokines, and n3PUFAs

Obesity in humans has been associated with the metabolic syndrome, which consists of a cluster of disorders including, insulin resistance and type II DM, dyslipidemia and hepatic steatosis, hypertension, and atherosclerosis and coronary heart disease. Obese humans are also more likely to suffer from certain cancers, osteoarthritis, and respiratory disorders. It is well-established that obese human subjects that suffer from the metabolic syndrome display a characteristic imbalance of their adipokine profile. This altered adipokine profile leads to profound changes in insulin sensitivity and other biochemical alterations of metabolites, making an individual more prone to metabolic disorders (Deng and Scherer, 2010).

Adiponectin deficiency is the most common adipokine aberration implicated in the development of the metabolic syndrome. Adiponectin knockout mice were shown to exhibit characteristics of the metabolic syndrome including insulin resistance, glucose intolerance, hyperlipidemia, and hypertension (Kadowaki and Yamauchi, 2005). Moreover, administration of exogenous adiponectin was demonstrated to ameliorate metabolic alterations associated with obesity in animal models (Yamauchi et al., 2001; Xu et al., 2003; Yamauchi et al., 2003b).

Both leptin deficiency and leptin resistance, leading to a state of reduced leptin function, have been argued to have an important role in the pathogenesis of the metabolic syndrome. Leptin treatment of leptin deficient mice (*ob/ob*), and restoring leptin receptor function in the brain of a mouse model of complete absence of leptin receptor function (*db/db*) yielded normalization of the metabolic phenotype (Harris et al., 1998; de Luca et al., 2005).

Dietary supplementation with long chain n3PUFAs in human subjects or animal models of the metabolic syndrome has been generally shown to ameliorate metabolic alterations

including reduction of insulin resistance, triglyceride concentrations, and blood pressure, and elevation of high density lipoprotein (HDL)- cholesterol concentrations (Sekiya et al., 2003; Carpentier et al., 2006). However, a recent study demonstrated a differential effect of n3PUFA supplementation on metabolic alterations in a rodent model of the metabolic syndrome compared to a healthy related line; serum concentrations of adiponectin were increased by n3PUFA supplementation in healthy but not in affected animals and serum concentrations of leptin were higher in affected animals on supplementation while no difference was revealed without supplementation (Burghardt et al., 2010). These findings suggest complex associations between n3PUFAs and adipokines in disease.

Obesity has been associated with DM, orthopedic and urinary tract diseases, and increased risk for neoplastic diseases in dogs and cats, and in addition, with HL in cats and hypothyroidism and hyperadrenocorticism in dogs (German, 2006). The occurrence of naturally-occurring metabolic syndrome related to obesity has not been described in dogs or cats. Nevertheless, some of the diseases associated with obesity in these species display similar characteristics to specific components of the metabolic syndrome in humans. Feline HL has common characteristics as human hepatic steatosis including hepatic lipid accrual as a major feature of the disease. A major cause of feline DM is obesity-related insulin resistance, similar to human type II DM. Insulin resistance induced by obesity is also an important complicating factor in canine DM, although practically all cases are caused by insulin deficiency. In canine hypothyroidism obesity is consequence of the primary pathology rather than a predisposing or complicating factor.

2.1.2. *Feline hepatic lipidosis*

Feline HL is a common and potentially fatal liver disorder. HL is a common disease in cats with a reported prevalence of 0.04% in normal-weight cats and up to 0.2% in obese cats (Lund et al., 2005). It is the most common feline liver disorder. In a recent 10-year retrospective study, HL accounted for 50% of all diagnoses of feline liver biopsies (Armstrong and Blanchard, 2009). The pathogenesis of this disease is still not completely understood; nevertheless, the most important identified risk factor is obesity (Center, 2005). It has been proposed that obesity predisposes cats to HL mainly due to the increased availability of free FAs that can be mobilized from peripheral fat stores during periods of reduced food intake. Additional potential contributing factors include preexisting insulin resistance (Hoenig et al., 2007) and higher baseline hepatic lipid content (Nicoll et al., 1998) related to obesity.

Alterations in lipid and glucose metabolism have been described both in naturally-occurring and experimentally-induced feline HL (Biourge et al., 1997; Pazak et al., 1998; Brown et al., 2000a; Blanchard et al., 2004). Affected cats had increased serum concentrations of nonesterified FAs, triglycerides, VLDL, and low density lipoproteins (LDL), as well as decreased insulin concentrations and reduced glucose tolerance.

Adiponectin and leptin have an important role in lipid metabolism, both directly and through their insulin-sensitizing effects. Most importantly, these adipokines decrease lipid content of non-adipose tissues. Leptin inhibits hepatic lipogenesis and enhances FA oxidation in skeletal muscle and liver (Yu and Ginsberg, 2005). Adiponectin inhibits FA uptake into the hepatocytes and facilitates their uptake into skeletal myocytes while enhancing FA oxidation in both tissues (Yamauchi et al., 2001; Aguilera et al., 2008). Obese and insulin-resistant cats have

decreased concentrations of adiponectin but increased concentrations of leptin, indicating leptin resistance (Backus et al., 2000; Appleton et al., 2002; Hoenig et al., 2007; Ishioka et al., 2009) similar to findings in other species (German et al., 2010).

In line with its anti-steatotic effect in the liver, adiponectin was effective in alleviating hepatic steatosis in a model of fatty liver in obese mice (Xu et al., 2003). Serum concentrations of adiponectin were found to be decreased in human patients with non-alcoholic fatty liver disease (NAFLD), which is characterized mainly by hepatic steatosis associated with obesity (Targher et al., 2004; Bugianesi et al., 2005; Yoneda et al., 2007; Schreuder et al., 2008), similar to feline HL. Therefore, adiponectin has been suggested to play an important role in the pathophysiology of the disease. The importance of leptin in lipid metabolism is exemplified by the development of hepatic steatosis in rodent models of either leptin deficiency or leptin resistance (Yu and Ginsberg, 2005). Yet, studies on leptin in human NAFLD have yielded less consistent results (Chalasani et al., 2003).

2.1.3 Canine hypothyroidism

Hypothyroidism is one of the most common endocrine diseases in dogs. Primary hypothyroidism is the most common cause of the naturally-occurring disease, accounting for more than 95% of the cases. Two histologic forms of primary hypothyroidism predominate including: lymphocytic thyroiditis and idiopathic thyroid atrophy; the end result of these 2 forms is progressive destruction of the thyroid gland and the resultant deficiency of circulating thyroid hormones (Feldman and Nelson, 2004c). Obesity is a main feature of the disease, occurring in 41-48% of the hypothyroid dogs (Panciera, 1994; Dixon et al., 1999). The disease has also been associated with alterations in lipid and carbohydrate metabolism.

Hyperlipidemia with increased concentrations of triglyceride and cholesterol occurs in 75% to 90% of the hypothyroid dogs (Panciera, 1994; Dixon et al., 1999). In contrast to the predominance of HDL that is characteristic to healthy dogs (Mahley et al., 1974), hyperlipidemia in hypothyroid dogs is associated with elevations in LDL, HDL1, and VLDL, resulting in a typical atherogenic lipoprotein profile (Barrie, et al. 1993). Indeed, although atherosclerosis is generally rare in dogs, its prevalence is likely higher in hypothyroid dogs. An association of atherosclerosis with hypothyroidism was first noted in a family of Beagles (Manning, 1979). Later, hypothyroidism was reported to be a common diagnosis in dogs showing atherosclerosis on necropsy (Liu et al., 1986), and dogs with atherosclerosis were found to be 51 times more likely to be hypothyroid compared to dogs without atherosclerosis (Hess et al., 2003).

Alterations in carbohydrate metabolism are far less well documented in canine hypothyroidism. The occurrence of insulin resistance was first suggested in radioiodine-induced hypothyroid dogs that had prolonged insulin response to intravenous glucose administration

compared to healthy controls (Renauld et al., 1982), and later in three dogs with naturally-occurring hypothyroidism and concurrent DM that required high doses of insulin prior to thyroxin replacement therapy (Ford et al., 1993).

Although obesity has been associated with hypoadiponectinemia in dogs, similar to findings in humans and rodents (Ishioka et al., 2006; Brunson et al., 2007) and obese dogs are reported to have similar lipoprotein changes to obese humans (Bailhache et al., 2003), in contrast to the predisposition of obese humans to develop atherosclerosis (Fantuzzi and Mazzone, 2007), obesity alone has not been associated with atherosclerosis in dogs. Indeed, in an early study, induction of hypothyroidism was required in order to create atherosclerosis in dogs fed a high-fat diet (Mahley et al., 1974).

2.1.4. *Canine and feline diabetes mellitus*

DM is the most common disorder of the endocrine pancreas in dogs and cats with reported frequency varying from 1 in 500 to 1 in 100 (Panciera et al., 1990). The prevalence of feline DM is reported to have increased dramatically over the past several decades (Pahl et al., 2007), parallel to the increase in the prevalence of the disease in humans. DM in humans is classified based on the pathophysiologic mechanism into type I, i.e. insulin deficiency, and type II, i.e. insulin resistance. A more clinical classification based on the clinical requirement in insulin for glycemic control is usually used in dogs, i.e. insulin dependent DM (IDDM) and non-insulin-dependent DM (NIDDM) (Feldman and Nelson, 2004a). Virtually all dogs have IDDM at the time of diagnosis and its etiology is multifactorial; potential factors include genetic predisposition, immune-mediated disease, and pancreatitis (Feldman and Nelson, 2004b). Clinical recognition of NIDDM is more frequent in cats, accounting for approximately 30% of the cats at the time of diagnosis; potential etiologic factors include islet amyloidosis, obesity and pancreatitis.

Alterations in carbohydrate metabolism are evident both in feline and canine DM and hyperglycemia is the mainstay of the disease, as expected. In dogs, the disease is characterized by hypoinsulinemia and essentially no increase in insulin concentrations following administration of an insulin secretagogue at any time following diagnosis of the disease. Diabetic cats, on the other hand, may have either increased or decreased concentrations of insulin and variable responses to an insulin secretagogue, depending on the etiology and stage of the disease and presence of glucose toxicity; a diabetic cat with concurrent disease and glucose toxicity at presentation may have decreased insulin concentration at first, but higher concentrations of

insulin once glucose toxicity resolved. On the other hand, a cat with type II DM may initially present with increased concentrations of insulin, but these concentrations may gradually decrease with progression of islet pathology and deterioration of insulin secretion capacity (Feldman and Nelson, 2004b).

Alterations in lipid metabolism are also common in canine and feline DM. Untreated or uncontrolled DM is accompanied by increases of triglyceride, cholesterol, lipoprotein, chylomicrons, and free FAs. Dogs with DM have been shown to have increased VLDL and HDL (Barrie et al., 1993). Moreover, dogs with atherosclerosis were demonstrated to be 53 times more likely to have concurrent DM than dogs without atherosclerosis (Hess et al., 2003).

2.1.5. Hypotheses

Obesity is an important predisposing factor for feline HL and feline DM and is also a major consequence of canine hypothyroidism, which is also associated with hyperlipidemia and atherosclerosis. In healthy dogs and cats, obesity is associated with adiponectin deficiency and leptin resistance.

In view of the anti-steatotic, insulin-sensitizing, hypolipidemic, and anti-atherogenic effects of these adipokines, we hypothesized that naturally-occurring feline HL, feline DM, and canine hypothyroidism are associated with dysfunction of these adipokines' metabolic pathways (either deficiency or resistance) that would be manifested in altered serum concentrations, independent of body condition.

Obesity may serve as a complicating factor in canine DM, but is not a major feature of the disease, which is caused by insulin deficiency. Due to the effects of insulin on adiponectin and leptin secretion, we hypothesized that naturally-occurring canine DM is associated with decreased leptin and increased adiponectin serum concentrations.

Due to the known beneficial effects of n3PUFAs on some of the metabolic alterations associated with these diseases in dogs and cats (i.e. insulin resistance, hyperlipidemia, hepatic steatosis, and atherosclerosis) and the associations of adipokines with these metabolic alterations as well, we hypothesized that circulating concentrations of n3PUFAs in cats with HL and DM and in dogs with hypothyroidism and DM are associated to concentrations of adipokines.

2.2. Studies: study aims, methods, and results

2.2.1. Circulating concentrations of adipokines and n3PUFAs in feline hepatic lipidosis

Study aims

- To determine the effect of feline HL on circulating concentrations of adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol by comparing serum concentrations among cats diagnosed with HL, cats diagnosed with other liver disease, and healthy cats.
- To determine the effect of n3PUFA on circulating concentrations of adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol in cats with HL.
- To compare serum concentrations of n3PUFAs between healthy cats and cats with HL.

Methods

Study design

Forty five cats with liver disease and 55 healthy adult cats were enrolled in the study. Client-owned cats with newly-diagnosed liver disease were recruited from the Veterinary Teaching Hospitals at Michigan State University (MSU) and at the Hebrew University of Jerusalem (HUJI) and were allocated into 1 of 3 groups: 1) HL alone (n=20), 2) HL and concurrent disease (n=19), and 3) Other liver disease (n=6). Diagnosis of liver disease was based on clinical signs and clinical pathology, abdominal ultrasound, and liver cytopathology and/or histopathology findings. Exclusion criteria included initiation of enteral feeding or parenteral nutrition, or administration of glucocorticoids prior to sample collection. The healthy cats were client-owned cats visiting MSU for routine check-up. The cats were considered to be healthy based on physical examination and routine biochemistry analysis. Additional inclusion criteria were based on medical and dietary history for a 3 month period preceding sample collection and included: 1) absence of any clinical signs indicating illness; 2) exclusive feeding of a commercially available, nutritionally balanced and complete adult cat diet, and; 3) no administration of dietary supplements or medications.

Body condition of all cats was evaluated by 2 individuals using a BCS system on a scale of 1- 9 (Laflamme, 1997a) and fasted serum samples were collected. The study was performed in compliance with Michigan State University and the Hebrew University of Jerusalem guidelines for research in animals. Informed consent was obtained from all owners.

Serum analysis

Serum was separated immediately following blood collection and frozen at -80°C until analyzed. Serum adiponectin, leptin and insulin concentrations were measured using commercially available assays previously used in cats (Backus et al., 2000; Hoenig et al., 2007) and validated in our laboratory (Mouse/rat Adiponectin ELISA kit-B-Bridge, Multispecies Leptin RIA kit- Millipore, Human Insulin RIA kit- Diagnostic Systems Laboratories- See Appendix C, Protocols 1 and 2). Serum concentrations of glucose, cholesterol and triglyceride were measured using a spectrophotometric method (Kodak Ektachem DT60 II Clinical Products Division, Eastman Kodak Co). Additional biochemistry parameters were measured using automated chemistry analyzers (AU640^e Chemistry-Immuno System, Olympus America- at MSU, Cobas Mira Chemistry Automatic Analyser, Roche- at HUJI). Serum FA analysis was performed by gas chromatography (See Appendix C, Protocol 3).

Data analysis

Associations between liver disease and the outcome variables (serum concentrations of adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol) were evaluated using general linear model (GLM) including group (healthy cats, cats with HL alone, cats with HL and concurrent disease, and cats with other liver disease) as factor , and BCS, age, gender, and location (MSU or HUJI) as covariates. The additional covariates were included to control for potential confounding of the relationship between liver disease and the outcome variables. The link function and the linear predictor for each of the variables are of the form $E[Y|x] = a + bx$. Y is a natural logarithmic transformation of the measured values and has a normal distribution (as confirmed by P–P plots) with a mean $(a + bx)$ and constant variances. All interaction effects

between group and the additional covariates were assessed. Covariates and interaction terms with significance level of effect $P \geq 0.1$ were removed from the models. Association between liver disease and adiponectin to leptin ratio was determined using GLM with group as a factor. Bonferroni adjustment was applied for multiple posthoc pairwise comparisons among the groups.

Model fitting and post post-hoc categorization- The covariates age and gender were removed from the models evaluating the associations between liver disease and all outcome variables. The covariate location was included in the model evaluating the association between liver disease and insulin due to a significant ($P < 0.001$) effect, but was removed from all other models. Interaction effects were present between BCS and group in the analyses of the association of liver disease and adiponectin ($P = 0.004$) or triglyceride ($P = 0.067$). Therefore separate analyses for body condition groups were performed for adiponectin and triglyceride. Due to insufficient statistical power of the study to evaluate the effect of total or individual n3PUFA on the outcome variables in separate analyses of each BCS level, cats were categorized into two groups. Categorization into non-obese (BCS 4-6) and obese (BCS 7-9) in the analysis of adiponectin was based on the presence of a significant effect of BCS on adiponectin in obese cats ($P=0.002$) and absence of such an effect in non-obese cats ($P = 0.1$). Categorization into lean (BCS 4-5) and overweight (BCS 6-9) in the analysis of triglyceride was based on the presence of a significant effect of BCS on triglyceride in overweight cats ($P = 0.028$) and absence of such an effect in the lean cats ($P = 0.3$).

When means of the outcome variable in the groups of cats with liver diseases (cats with HL alone, cats with HL and concurrent disease, and cats with other liver disease) were all either higher or lower compared to healthy cats in the analyses described above, further analyses of the

associations between liver disease and the outcome variable were performed using all cats with liver disease grouped together and healthy cats as the group factor.

Linear correlations between the outcome variables and BCS were evaluated using Spearman correlation coefficient. Linear correlations between the outcome variables and fold increases in serum concentrations of total bilirubin or albumin, or fold increases of serum activities of ALT, AST, and ALP were evaluated using Pearson correlation coefficient. Natural log transformation was used for any non-normally distributed variable (all variables but total bilirubin).

Associations between serum concentrations of total n3PUFAs or serum concentrations of individual n3PUFA (EPA, DPA, DHA, and ALA) and serum concentrations of adiponectin, leptin, insulin, glucose, triglyceride, or cholesterol within the group of cats with HL were evaluated using GLM including total n3PUFAs or individual n3PUFAs, BCS, gender, and age as covariates. Absence of potential collinearity among serum concentrations of EPA, DPA, DHA, and ALA was confirmed. The additional covariates (BCS, gender, and age) were included to control for potential confounding of the relationship between n3PUFA and the outcome variables (adiponectin, leptin, insulin, glucose, triglyceride, or cholesterol). The link function and the linear predictor for each of the variables are of the form $E[Y|x] = a + bx$. Y is a natural logarithmic transformation of the measured values and has a normal distribution (as confirmed by P–P plots) with a mean ($a + bx$) and constant variances. All interaction effects between n3PUFAs and other variables in the models were assessed.

Model fitting and post hoc categorization - Significant interaction effects between BCS and total n3PUFAs were present in the analyses of the associations with adiponectin ($P = 0.037$), triglyceride ($P = 0.002$), and glucose ($P = 0.048$). A nearly significant interaction effect between

BCS and total n3PUFAs was present in the analysis of the associations with leptin ($P = 0.069$). Due to insufficient statistical power of the study to evaluate the effect of total or individual n3PUFA on the outcome variables in separate analyses of each BCS level, cats were categorized into two groups (non-obese: BCS 4-6 and obese: BCS 7-9), similar to the categorization used for healthy cats. Results are reported separately for the non-obese and obese groups. The covariates age and gender were removed from all models due to insignificant effect ($P \geq 1.0$)

Linear correlations between serum concentrations of total or individual n3PUFAs and serum activities of liver enzymes or concentration of bilirubin within cats with liver disease were evaluated using Spearman correlation coefficient.

Mann-Whitney U test was used to compare serum concentrations of FAs between healthy cats and cats with HL.

Data were analyzed using a commercially available statistic program (SPSS 19.0 for Windows). $P < 0.05$ was considered statistically significant.

Results

Cats

The healthy cats group consisted of 55 healthy cats included in study 1.2.3. The liver disease group included 23 neutered females, 20 neutered males, 1 intact female and 1 intact male. Age ranged from 2 to 17 (mean: 7.6 y). BCS ranged from 2 to 9 (Median: 6). Breeds among the liver disease group consisted of domestic short hair (29), domestic long hair (9), mixed breed (4), Maine Coon (1), and Siberian (1). Concurrent diseases in cats with HL included

pancreatitis, cholangiohepatitis, cholecystitis, lymphoma, and organic phosphate toxicity. Other liver diseases included suppurative cholangiohepatitis and lymphosarcoma. Changes in biochemistry parameters in cats with liver disease are presented in Table 20.

Table 20. Biochemical changes in cats with liver diseases.*

Variable	HL	HL and concurrent disease	Other liver disease
Blood urea nitrogen	0.5 (0.2, 1.0)	0.5 (0.2, 1.1)	0.4 (0.3, 0.9)
Creatinine	0.5 (0.2, 1.0)	0.6 (0.4, 1.1)	0.6 (0.1, 0.8)
Albumin	0.7 (0.5, 1.0)	0.7 (0.4,1.0)	0.7 (0.5, 0.8)
Total bilirubin	19.3 (0.3, 34.1)	14.6 (0.2, 36.1)	4.2 (1.5, 30.2)
Alkaline phosphatase	7.9 (0.1, 46.6)	3.5 (0.1, 23.7)	0.5 (0.1, 6.7)
Alanine aminotransferase	1.4 (0.5, 10.4)	2.1 (0.4, 18.1)	2.1 (1.3, 8.9)
Aspartate aminotransferase	1.9 (0.1, 6.3)	2.2 (0.1, 31.4)	2.6 (0.2, 6.1)

* Values presented are median (range) of fold increase of the upper reference range value.

Associations between liver disease and serum concentrations of hormones, lipids, and glucose

Adjusted serum concentrations of hormones, lipids, and glucose in healthy cats, cats with HL, cats with HL and concurrent disease, and cats with other liver disease are presented in Table 21 and the measured values of serum concentrations are presented in Figure 12.

Serum concentrations of adiponectin in obese cats were significantly higher in cats with HL alone or with concurrent disease than in healthy cats. Serum concentrations of leptin in cats with HL alone or with concurrent disease were significantly higher than concentrations in healthy cats and cats with other liver disease. Serum concentrations of insulin were significantly

lower in cats with other liver disease than healthy cats. Concentrations of glucose in cats with HL alone and cats with HL and concurrent disease were higher than healthy cats. Concentrations of triglyceride were significantly higher in cats with HL alone than healthy cats both in lean and obese cats. In lean cats, concentrations of triglyceride in cats with HL alone were also significantly higher than cats with HL and concurrent disease or cats with other liver disease (Table 21).

When all cats with liver disease were grouped together, serum concentrations of adiponectin (adjusted for BCS in the obese group) were significantly higher compared to healthy cats both in the obese (4.4 $\mu\text{g/mL}$; 3.1, 6.1 vs. 1.2 $\mu\text{g/mL}$; 0.9, 1.6, $P < 0.001$) and non-obese (4.2 $\mu\text{g/mL}$; 3.1, 5.5 vs. 2.4 $\mu\text{g/mL}$; 1.9, 3.1, $P = 0.005$) cats. Serum concentrations of insulin (adjusted to BCS and location) were significantly ($P = 0.042$) lower in cats with liver disease (22 pmol/L; 18, 26) compared to the healthy cats (29 pmol/L; 23, 36). Serum concentrations of glucose were significantly ($P < 0.001$) higher in cats with liver disease (135 mg/dL; 124, 146) compared to healthy cats (100 mg/dL; 92, 107). Serum concentrations of cholesterol (adjusted to BCS) were significantly ($P = 0.027$) lower in cats with liver disease (115 mg/dL; 104, 128) compared to healthy cats (136 mg/dL; 123, 149).

Table 21. Serum concentrations of hormones, glucose, and lipids in cats with liver diseases and healthy cats.*

Variable	Covariates†	Group			
		Healthy‡	HL‡	HL with concurrent disease‡	Other liver disease
Adiponectin (µg/mL)					
Non-obese	-	2.4 (1.9, 3.1)	3.6 (2.3, 5.7)	4.2 (2.7, 6.3)	6.0 (2.9, 12.4)
Obese	BCS	1.2 (0.9, 1.6) ^{a,b}	4.7 (2.8, 7.7) ^a	3.9 (2.2, 7.0) ^b	4.8 (1.7, 14.1)
Leptin (ng/mL)					
	BCS	4.9 (4.3, 5.5) ^{a,b}	9.8 (8.0, 12.0) ^{a,c}	10.7 (8.7, 13.2) ^{b,d}	4.9 (3.5, 7.1) ^{c,d}
Insulin (pmol/L)					
	BCS, Location	28 (22, 35) ^e	22 (17, 28)	25 (19, 32)	13 (8, 21) ^e
Glucose (mg/dL)					
	-	100 (92, 107) ^{a,b}	134 (118, 152) ^a	140 (123, 159) ^b	122 (97, 153)
Triglyceride (mg/dL)					
Lean	-	63 (51, 77) ^a	204 (141, 294) ^{a,b,e}	98 (75, 129) ^{c,e}	39 (25, 61) ^{b,c}
Overweight	BCS	91 (75, 109) ^e	152 (113, 204) ^e	119 (79, 181)	87 (40, 190)
Cholesterol (mg/dL)					
	BCS	136 (123, 149)	126 (108, 148)	104 (88, 123)	115 (86, 154)

* Associations between group and serum concentrations of hormones, glucose, or lipids were determined using GLM. †Covariates BCS, age, gender, and location and interaction terms between group and each of the covariates were evaluated in each model.

Adiponectin and triglyceride were analyzed in separate body condition groups due to significant interaction terms between BCS and groups. Covariates with an effect of significance level $P \leq 0.1$ were included in each model. ‡ Adjusted mean (95% CI); Same letter within a row indicates a significant difference with Bonferroni correction for multiple comparisons: a, b, c, d- $P < 0.01$; e- $P < 0.05$.

Figure 12. Serum concentrations of adiponectin (A), leptin (B), insulin (C), glucose (D), triglyceride (E), and cholesterol (F) in healthy cats (□), cats with HL alone (◻), cats with HL and concurrent disease (◼), and cats with other liver disease (■).†

† Serum concentrations are presented on a natural logarithmic scale. The horizontal bar within the box plot indicates the median, the box contains the middle half of the results, the T-bars indicate the range, and the circles and stars indicate outlying and extreme data. Lean- BCS: 3-5; Overweight- BCS:6-9; Non-obese- BCS:3-6; Obese- BCS:7-9.

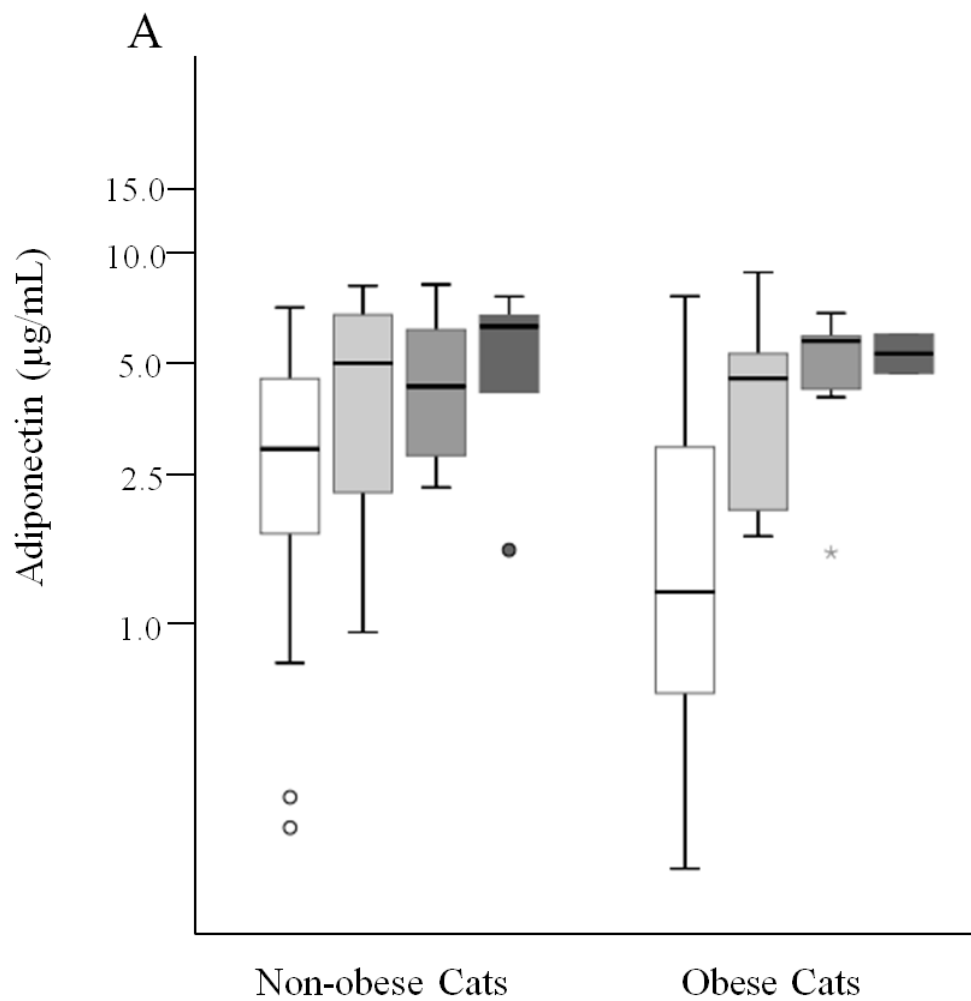


Figure 12 (cont'd)

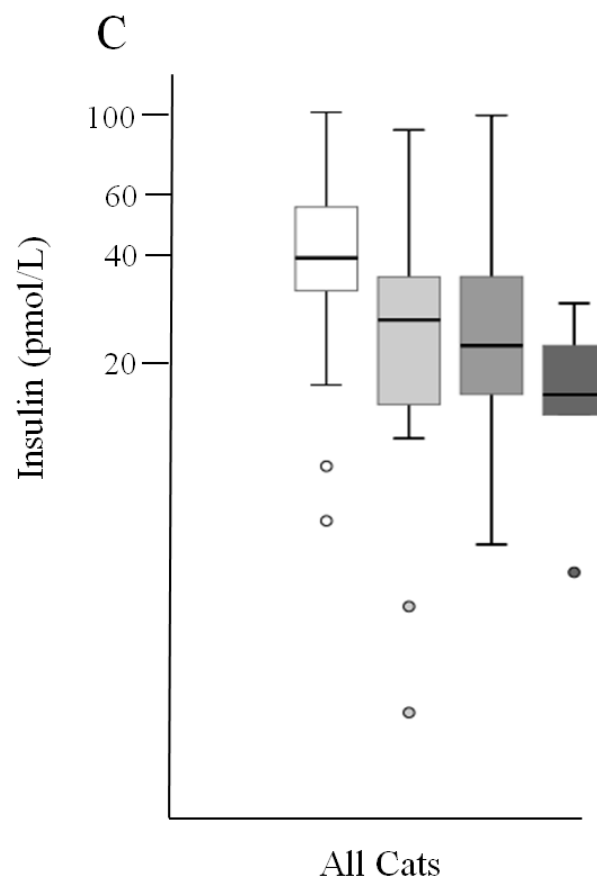
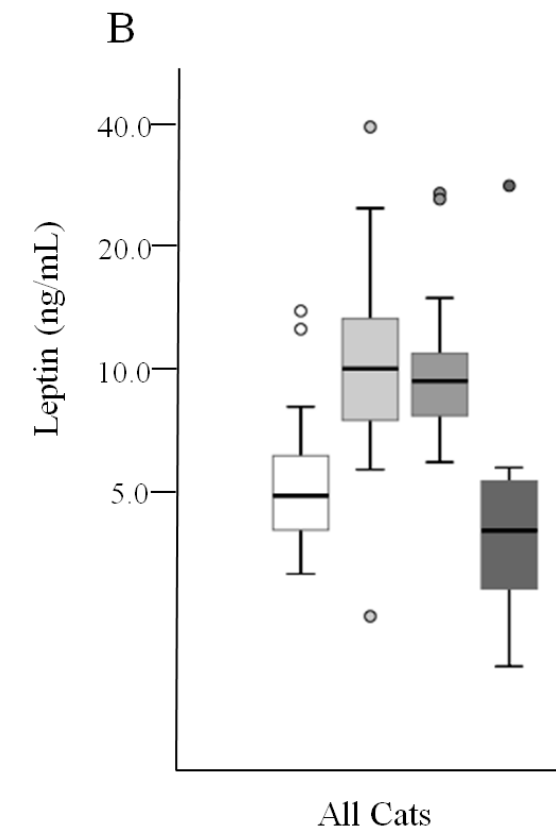


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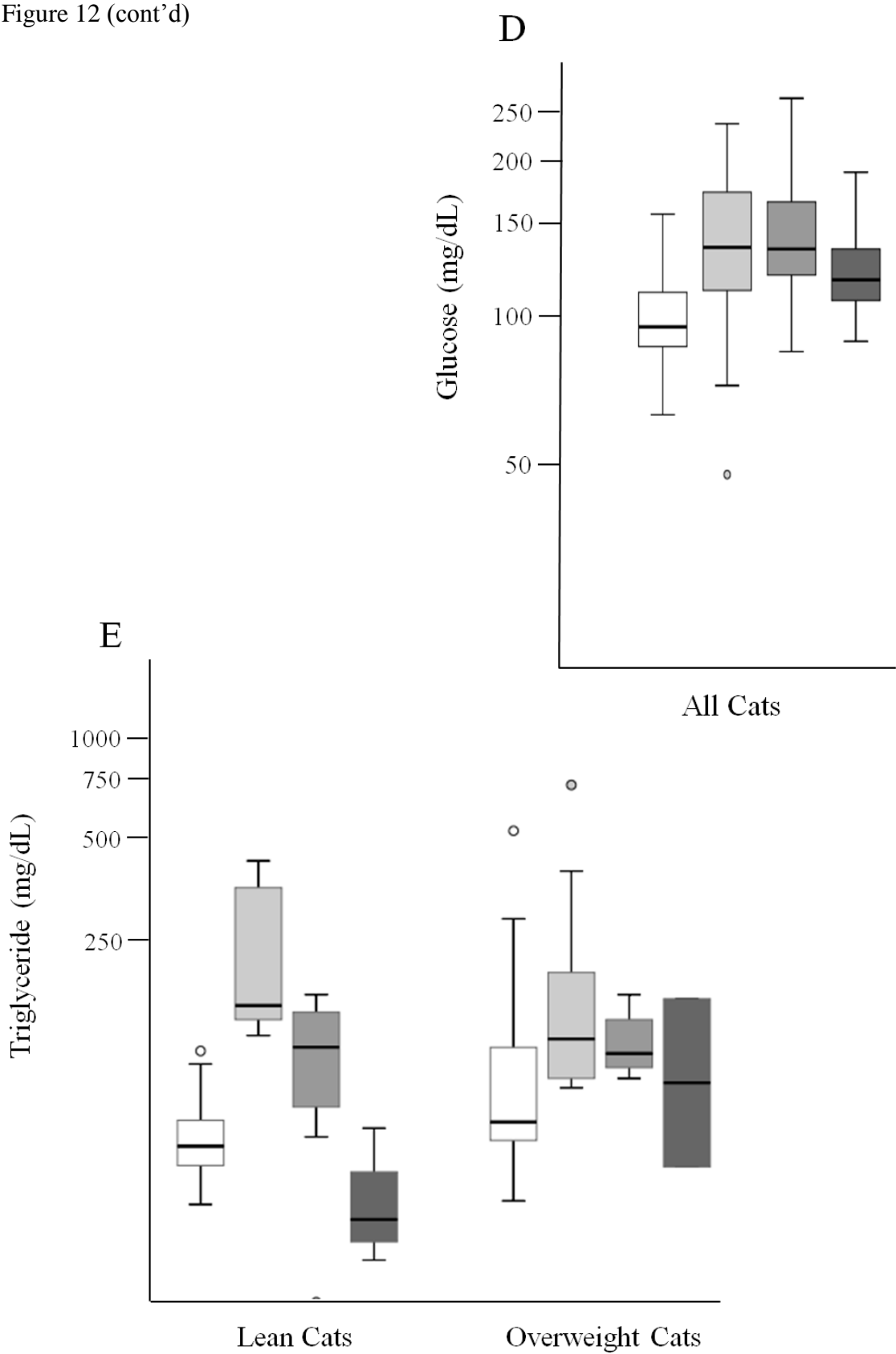
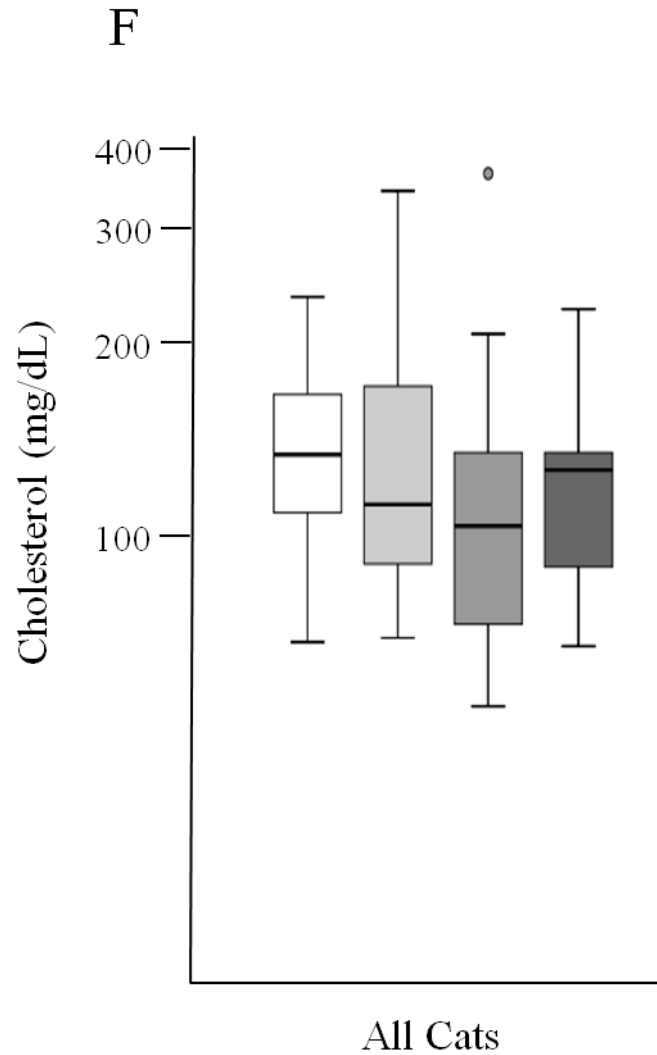


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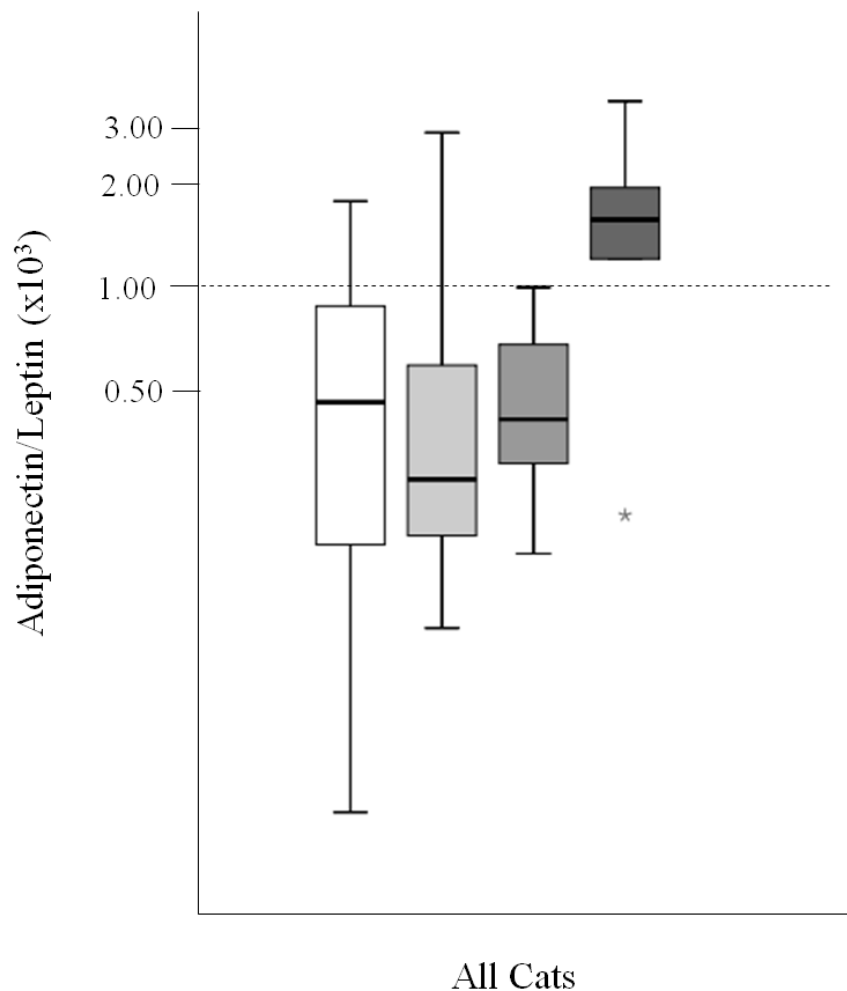
Adiponectin to leptin ratio in cats with liver disease

The ratio of serum concentrations of adiponectin to serum concentration of leptin (Mean; (95% CI) was significantly higher in cats with other liver disease (1.26×10^3 ; 0.57×10^3 , 2.78×10^3) compared to cats with HL alone (0.35×10^3 ; 0.22×10^3 , 0.55×10^3 ; $P = 0.006$), cats HL

with concurrent disease (0.41×10^3 ; 0.26×10^3 , 0.65×10^3 ; $P = 0.017$), or healthy cats (0.37×10^3 ; 0.29×10^3 , 0.49×10^3 ; $P = 0.005$, respectively), (Figure 13).

Figure 13. Adiponectin to leptin ratio in healthy cats (□), cats with HL alone (▨), cats with HL and concurrent disease (▩), and cats with other liver disease (■).†

† The ratio is presented on a natural logarithmic scale. The horizontal bar within the box plot indicates the median, the box contains the middle half of the results, the T-bars indicate the range, and the star indicates extreme data point.



Associations among BCS and serum concentrations of hormones, lipids, and glucose in cats with liver disease

In cats with liver disease, BCS was significantly positively correlated to serum concentrations of leptin ($r = 0.38$, $P = 0.013$) only.

No significant linear correlations were present among adiponectin, leptin, and insulin. Serum concentrations of triglyceride were significantly positively correlated to leptin ($r = 0.56$, $P < 0.001$) and insulin ($r = 0.35$; $P = 0.02$).

Associations between hormones, lipids, and glucose and biochemical parameters among cats with liver disease

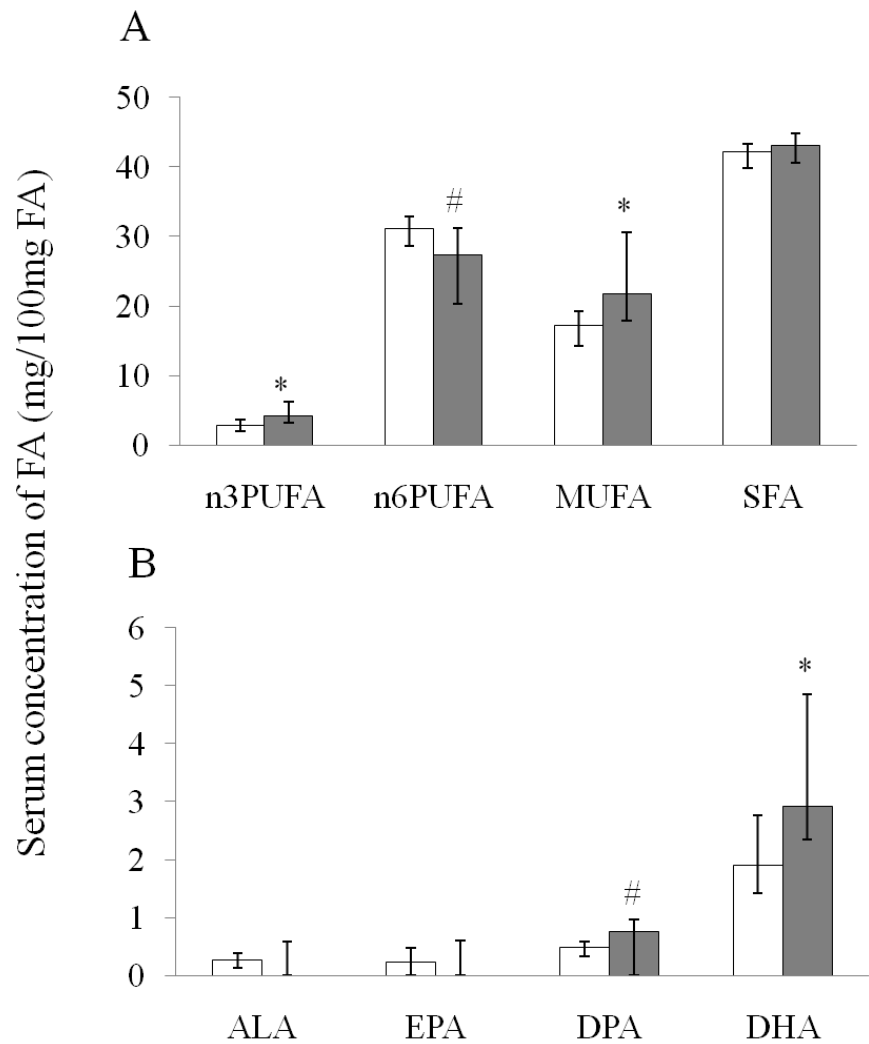
When all cats with liver disease were grouped together, serum concentrations of leptin were significantly positively correlated to fold increase in serum ALP activity ($r = 0.42$, $P = 0.005$). Serum concentrations of adiponectin were significantly positively correlated fold increase in serum ALT activity ($r = 0.40$, $P = 0.007$).

Serum concentrations of FAs in healthy cats and cats with HL

Serum FA analysis was available for 21 cats with HL (11 non-obese and 10 obese) and all healthy cats. Cats with HL had significantly higher serum concentrations of n3PUFAs and MUFAs and significantly lower concentrations of n6PUFAs than healthy cats. Of the individual n3PUFAs, serum concentrations of DPA and DHA were significantly higher in cats with HL (Figure 14 A and B). No significant differences were present between serum concentrations of FAs between non-obese and obese cats within the healthy or HL groups.

Figure 14. Serum concentration of total FAs within categories (A) and individual n3PUFAs (B) in healthy cats (□) and cats with HL (■).†

† Bar represents the median and error bars represent interquartile range * $P < 0.001$, # $P < 0.05$ compared to healthy cats.



Associations between concentrations of total n3PUFAs and concentrations of hormones, lipids, and glucose in cats with HL

In obese cats with HL, an increase of 1 mg/100mg FA in serum concentration of total n3PUFA was associated with a 17% decrease in the adjusted geometric mean of serum concentration of adiponectin, and a 24% decrease in the adjusted geometric mean of serum concentration of triglyceride (Table 22). No significant associations were present between concentration of total n3PUFAs and any of the outcome variables in non-obese cats.

Table 22. Associations between total n3PUFAs and adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol in 10 obese cats with HL.*

Variable	Mean (95% CI) †	Fold increase (95% CI) ‡	P	Partial η^2
Adiponectin	3.8 µg/mL (2.6, 5.7)	0.83 (0.69, 0.99)	0.032	0.51
Leptin	12.7 ng/mL (8.6, 18.7)	0.88 (0.72, 1.08)	0.2	0.20
Insulin	23 pmol/L (14, 40)	0.85 (0.64, 1.13)	0.2	0.18
Glucose	7.1 mmol/L (5.2, 9.7)	1.12 (0.95, 1.31)	0.2	0.23
Triglyceride	157 mg/dL (110, 225)	0.76 (0.63, 0.91)	0.008	0.66
Cholesterol	97 mg/dL (73, 129)	1.02 (0.88, 1.19)	0.7	0.01

* Covariates in the GLM include total n3PUFAs and BCS.

† Covariates in the model are evaluated at the following values: total n3PUFAs = 4.33 mg/100mg FA and BCS = 7.5.

‡ Fold increases and 95% CI in adjusted geometric means of serum concentration of each variable with an increase of 1 mg/100 mg FA in serum concentration of total n3PUFAs.

Associations between concentrations of individual n3PUFAs and concentrations of hormones, lipids, and glucose in cats with HL

In non-obese cats with HL, an increase of 1 mg/100mg FA in serum concentration of DHA was associated with a 27% decrease in the adjusted geometric mean of serum concentration of adiponectin (95% CI of fold increase of adjusted geometric mean: 0.56, 0.95; $P=0.025$; Partial $\eta^2 = 0.59$) while there was a tendency for an opposite association between EPA and adiponectin concentrations; an increase of 1 mg/100mg FA in serum concentration of EPA was associated with a 23% increase in the adjusted geometric mean of serum concentration of adiponectin (95% CI of fold increase of adjusted geometric mean: 0.98, 1.55; $P = 0.068$; Partial $\eta^2 = 0.45$). No significant associations were present between the other outcome variables and any of the individual n3PUFAs in non-obese cats.

In obese cats with HL, an increase of 1 mg/100mg FA in serum concentration of DPA was associated with a 68% decrease in the adjusted geometric mean of serum concentration of adiponectin (95% CI of fold increase of adjusted geometric mean: 0.81, 0.89; $P=0.037$; Partial $\eta^2 = 0.70$). No significant associations were present between the other outcome variables and any of the individual n3PUFAs in obese cats.

Associations between concentrations of total or individual n3PUFAs and biochemical parameters in cats with HL

In obese cats with HL, concentrations of total n3PUFAs were significantly correlated to fold increase in ALT activity ($r = -0.78$, $P = 0.008$) and tended to correlate to fold increase in ALP activity ($r = -0.62$, $P = 0.054$), concentrations of DHA were significantly correlated to fold

increase in ALT activity ($r = -0.63$, $P = 0.05$) and tended to correlate to fold increase in ALP activity ($r = -0.59$, $P = 0.074$, and concentrations of ALA were significantly correlated to fold increase in ALP activity ($r = -0.66$, $P = 0.039$). No significant linear correlations between serum concentrations of total or individual n3PUFAs and liver enzymes were present in non-obese cats with HL.

2.2.2. Circulating concentrations of adipokines and n3PUFAs in canine hypothyroidism

Study aims

- To determine the effect of canine hypothyroidism on circulating concentrations of adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol by comparing serum concentrations of between dogs diagnosed with hypothyroidism and healthy dogs.
- To determine the effect of n3PUFAs on circulating concentrations of adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol in dogs with hypothyroidism.
- To compare serum concentrations of n3PUFAs between healthy dogs and dogs with hypothyroidism.

Methods

Study design

Twenty-five newly-diagnosed hypothyroid dogs and 25 healthy dogs matched by gender and within similar ranges of body condition and age were enrolled in the study. The hypothyroid dogs were identified from samples submitted to a referral veterinary diagnostic laboratory.

Thyroid evaluation included total thyroxine (Total T4; Immulite, Canine Total T4, Diagnostic Products Corporation) and Canine Thyroid Stimulating Hormone (cTSH; Immulite, Canine TSH, Diagnostic Products Corporation). All newly-diagnosed hypothyroid dogs were included in the study group if the following inclusion criteria were met: 1) a combination of decreased Total T4 (<10 nmol/L) and increased cTSH (>26 mU/L) serum concentrations; 2) clinical suspicion of hypothyroidism and absence of other illness based on clinical signs and clinicopathologic findings; and 3) available fasted blood samples prior to thyroid replacement therapy initiation. The normal group included healthy euthyroid dogs based on clinical signs, clinicopathologic findings, and Total T4 results within the reference range (13–51 nmol/L). The normal dogs were selected from the population of client-owned dogs from veterinary practices served by the laboratory during the same time period. Dogs medicated with steroids, trimethoprim-sulfonamide antibiotics or phenobarbital during the preceding month, were excluded from the study.

Body condition of all dogs was evaluated by 2 individuals using BCS system on a 1-9 scale (Laflamme, 1997b) and fasted serum samples were collected. The study was performed in compliance with the university guidelines for research on animals. Informed consent was obtained from all owners.

Serum analysis

Samples were separated and serum frozen at -80 °C. Concentrations of leptin and insulin were measured in all samples using commercially available assays (Porcine Leptin Active IRMA and Insulin RIA, Diagnostic Systems Laboratories- See Appendix C, protocol 2), previously validated for dogs. Serum concentrations of adiponectin were measured in 18 hypothyroid and 18 matched healthy dogs using a commercially available assay according to the manufacturer instructions (Canine Adiponectin ELISA, Millipore- See Appendix C, protocol 1). Cholesterol, triglyceride, and glucose concentrations were measured in all dogs using an automatic analyzer (Cobas Mira Chemistry Automatic Analyzer, Roche). Serum total lipid content measurement and FA analysis were performed by gas chromatography (See Appendix C, Protocol 3)

Data analysis

Associations between hypothyroidism and serum concentrations of adiponectin, leptin, or insulin were evaluated using a GLM including group (hypothyroidism or healthy) as a factor and BCS, gender, and age as covariates. Triglycerides, and insulin or leptin were entered as additional covariates in the analyses of leptin and insulin, respectively. The additional covariates (BCS, gender, age, triglycerides, leptin or insulin) were included to control for potential confounding of the relationship between hypothyroidism and adiponectin, leptin, or insulin. Cholesterol concentration was not entered as a covariate due to its high correlation to triglyceride and the possible collinearity between the 2 variables. Associations between hypothyroidism and concentrations of cholesterol, triglycerides, or glucose were evaluated by GLM including hypothyroidism and BCS as covariates. BCS was included as a covariate in these analyses for potential confounding of the relationship between hypothyroidism and concentrations of

cholesterol, triglycerides, and glucose because obesity may have an effect on these variables. The link function and the linear predictor for each of the variables are of the form $E[Y|x] = \alpha + \beta x$. Y has a normal distribution with a mean $(\alpha + \beta x)$. For some of the dependent variables (adiponectin, leptin, insulin, cholesterol, and triglyceride), Y is a logarithmic transformation of the measured values. This transformation was performed due to inequality of variance of these variables as well as inequality of variance of the residuals across the range of the fitted values. The log transformed variables as well as the measured values of glucose concentrations had normal distributions (as confirmed by P-P plots) and constant variances. All interaction effects between hypothyroidism and other variables in the models were assessed. Association between hypothyroidism and adiponectin to leptin ratio was determined using GLM with group as a factor.

Model fitting and post-hoc categorization- All covariates were retained in the models. There was a nearly significant ($P = 0.051$) interaction with BCS in the analysis of the association between hypothyroidism and leptin. Therefore, separate analyses were performed for overweight (BCS: 6-7) and obese (BCS:8-9) dogs. Analysis for lean to ideal body condition (BCS: 4-5) could not be completed due to the small number of dogs in this group. Results are reported separately for each body condition group for the analysis of leptin and for all dogs together for the analyses of the other outcome variables.

Linear correlations between the variables were examined by Pearson (serum cholesterol, triglycerides, glucose, total T_4 , cTSH, adiponectin, leptin, or insulin concentrations) or Spearman (BCS) correlation coefficients. Natural log transformation was used for adiponectin, triglycerides, and total T_4 to obtain normal distribution (as confirmed by P-P plots).

Associations between serum concentrations of n3PUFAs or serum concentrations of individual n3PUFAs (EPA, DPA, DHA, and ALA) and serum concentrations of adiponectin, leptin, insulin, glucose, triglyceride, or cholesterol were evaluated using GLM including n3PUFAs or individual n3PUFA, BCS, gender, and age as covariates. Absence of potential collinearity among serum concentrations of EPA, DPA, DHA, and ALA was confirmed. The additional covariates (BCS, gender, and age) were included to control for potential confounding of the relationship between n3PUFA and the outcome variables (adiponectin, leptin, insulin, glucose, triglyceride, or cholesterol). The link function and the linear predictor for each of the variables are of the form $E[Y|x] = a + bx$. Y is a natural logarithmic transformation of the measured values and has a normal distribution (as confirmed by P–P plots) with a mean ($a + bx$) and constant variances. All interaction effects between n3PUFAs and other variables in the models were assessed.

Model fitting and post-hoc categorization- The covariates age and gender were removed from all models due to insignificant effect ($P \geq 1.0$). No significant interaction effects were present between BCS and total n3PUFAs or any individual n3PUFAs.

Linear correlations between serum concentrations of total or individual n3PUFAs and serum activities of liver enzymes or concentration of bilirubin were evaluated using Spearman correlation coefficient.

Mann-Whitney U test was used to compare serum concentrations of FAs between healthy and hypothyroid dogs.

Data were analyzed using SPSS 19.0 for Windows. $P \leq 0.05$ was considered statistically significant.

Results

Dogs

The hypothyroid and healthy dogs groups each included 13 spayed females, 5 neutered males, and 7 intact males. Median (range) BCS and age were 6 (4-9) and 7.0 years (3.0-15.0) in the healthy dogs group and 7 (3-9) and 9.0 years (3.0-15.0) in the hypothyroid dogs group. The hypothyroid and healthy dog groups used for adiponectin measurements each included 8 spayed females, 4 neutered males, and 6 intact males. Median (range) BCS and age were 7 (5-9) and 8.0 years (4.0-12.0) in the healthy dogs group and 7 (4-9) and 8.5 years (3.0-15.0) in the hypothyroid dogs group. The healthy dog group included 14 mixed breed dogs, 2 Labrador retriever, 2 Pekingese, 2 boxers, and 1 of each of the following: golden retriever, beagle, American Staffordshire terrier, Border collie, and Great Dane. The hypothyroid group included 14 mixed breed dogs, 4 Labrador retriever, and 1 of each of the following: golden retriever, German shepherd, Dalmatian, Komondor, American pit bull terrier, American cocker spaniel, and English setter. Biochemical changes in the hypothyroid dogs are presents in Table 23.

Table 23. Biochemical changes in dogs with hypothyroidism.*

Variable	HT
Blood urea nitrogen	0.6 (0.2, 1.3)
Creatinine	0.7 (0.0, 1.1)
Albumin	0.9 (0.7, 1.3)
Total bilirubin	0.4 (0.0, 0.8)
Alkaline phosphatase	0.7 (0.3, 5.1)
Alanine aminotransferase	0.6 (0.2, 6.5)
Aspartate aminotransferase	0.8 (0.4, 2.3)

Table 23 (cont'd)

* Values presented are median (range) of fold increases of measured values of serum concentrations compared to the upper end of the reference range.

Associations between hypothyroidism and serum concentrations of hormones, lipids, and glucose

Adjusted serum concentrations of hormones, lipids, and glucose in healthy dogs and dogs with hypothyroidism are presented in Table 24 and the measured values of serum concentrations are presents in Figure 15.

Concentrations of leptin were significantly higher in hypothyroid compared to healthy dogs in overweight but not in obese dogs. Concentrations of adiponectin and insulin were significantly higher in hypothyroid than healthy dogs (Table 24). These differences in leptin, adiponectin, and insulin remained significant with inclusion of triglyceride and insulin as additional covariates ($P = 0.003$, $P = 0.04$, $P = 0.001$, respectively). Concentrations of triglyceride and cholesterol were significantly higher in hypothyroid than healthy dogs. No significant difference in concentrations of glucose was present between the two groups (Table 24).

Table 24. Serum concentrations of hormones, glucose, and lipids in dogs with hypothyroidism and healthy dogs.*

Variable	Covariates†	Healthy‡	Hypothyroid‡	P
Adiponectin (µg/mL)	BCS, Age, Gender	8.0 (5.6, 11.4)	17.2 (12.1, 20.5)	0.003
Leptin (ng/mL)				
Overweight	Age, Gender	4.9 (3.4, 7.0)	17.1 (11.2, 26.2)	<0.001
Obese	Age, Gender	23.1 (11.5, 46.2)	33.9 (21.3, 53.9)	0.2
Insulin (pmol/L)	BCS, Age, Gender	178 (124, 255)	427 (305, 1031)	0.001
Glucose (mg/dL)	BCS	91 (84, 99)	90 (83, 97)	0.8
Triglyceride (mg/dL)	BCS	78 (56, 108)	255 (186, 351)	<0.001
Cholesterol (mg/dL)	BCS	224 (183, 274)	566 (469, 683)	<0.001

* Associations between group and serum concentrations of hormones, glucose, or lipids were determined using GLM. †Covariates BCS, age, gender, and location as well as interaction terms between group and each of the covariates were evaluated in each model. Leptin was analyzed separately in different body condition groups due to significant interaction terms between BCS and groups. Covariates with an effect of significance level $P \leq 0.1$ were included in each model. ‡ Adjusted mean (95% CI).

Figure 15. Serum concentrations of adiponectin (A), leptin (B), insulin (C), glucose (D), triglyceride (E), and cholesterol (F) in healthy (\square) and hypothyroid (\blacksquare) dogs.[†]

[†] Serum concentrations are presented on a natural logarithmic scale. The horizontal bar within the box plot indicates the median, the box contains the middle half of the results, the T-bars indicate the range, and the circles indicate outlying data. Lean: BCS: 3-5, Overweight: BCS: 6-7, Obese: BCS: 8-9.

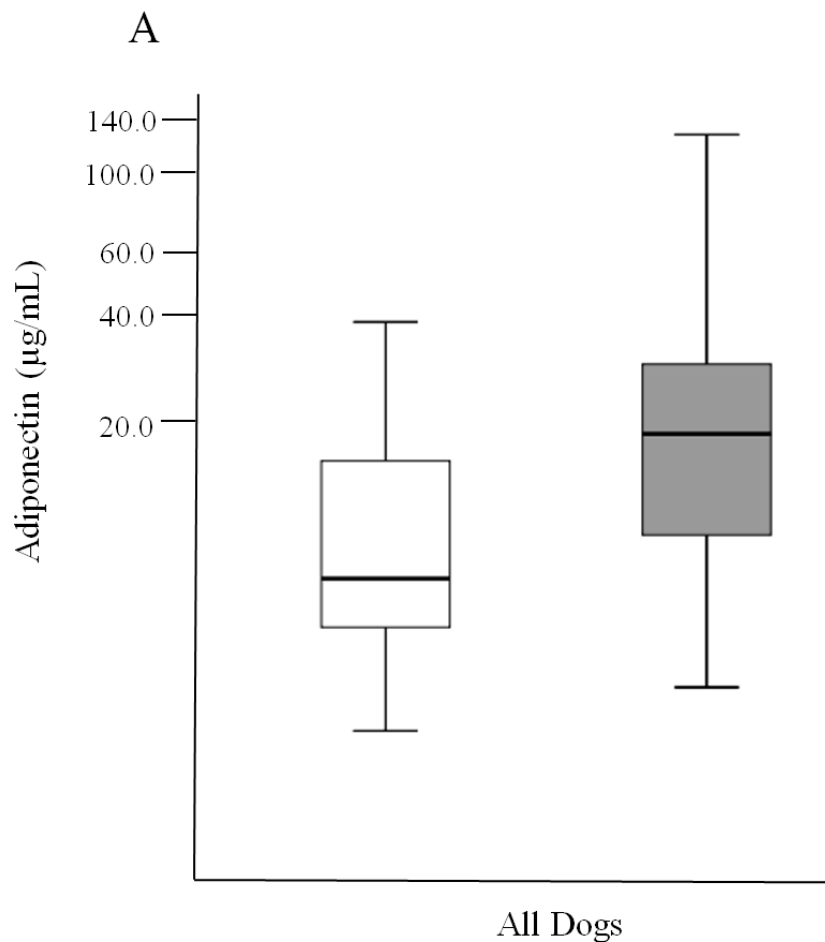


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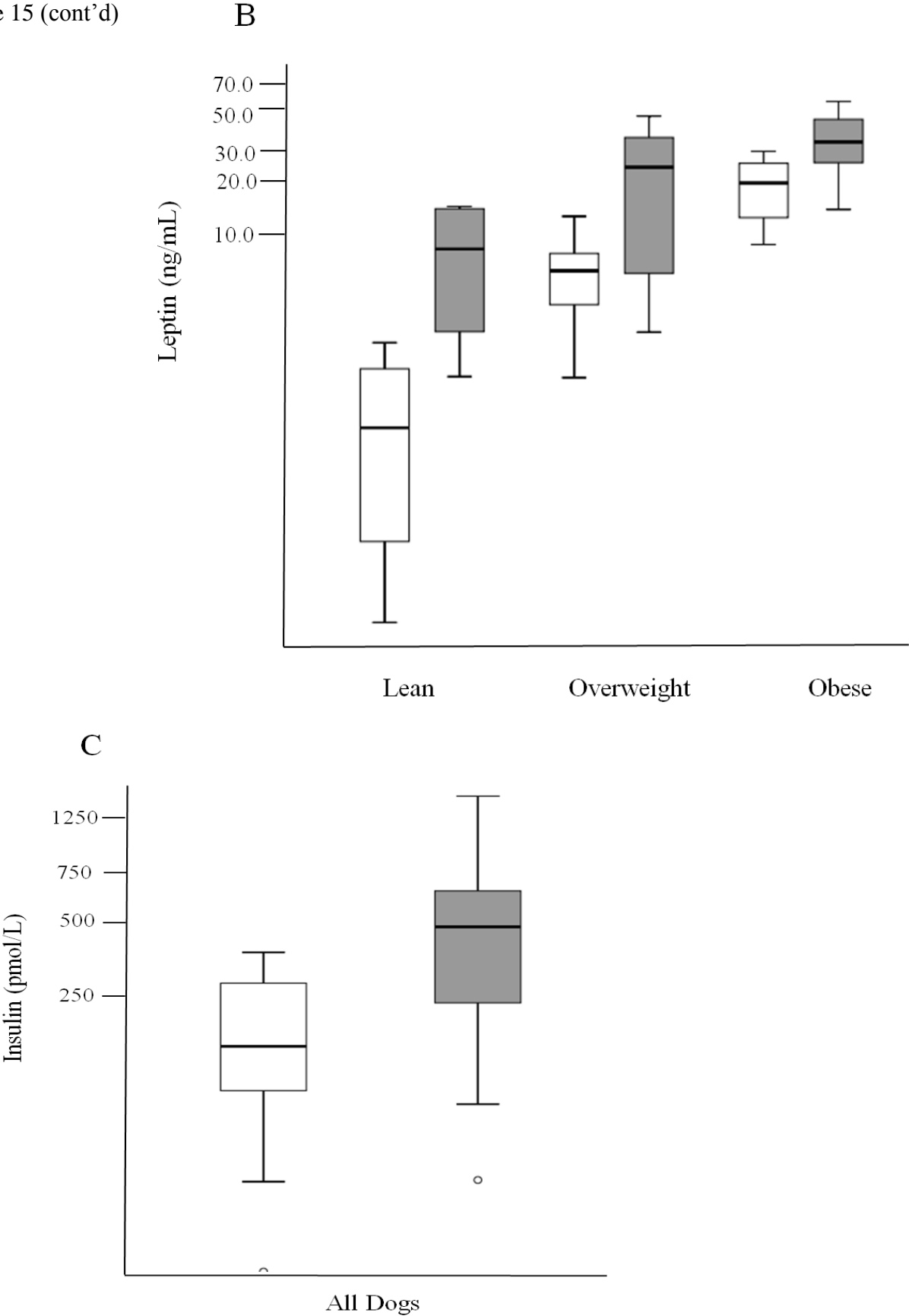


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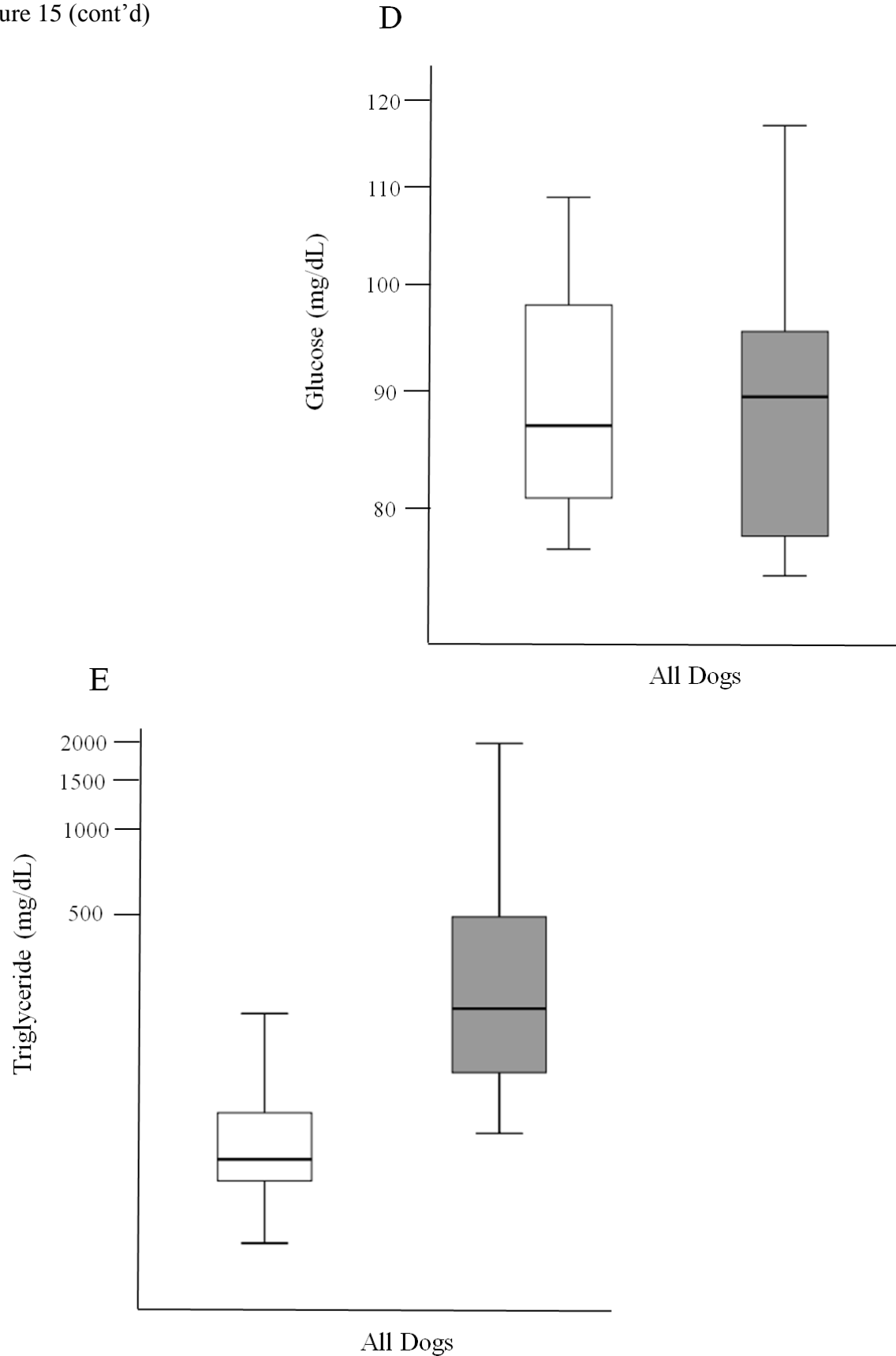
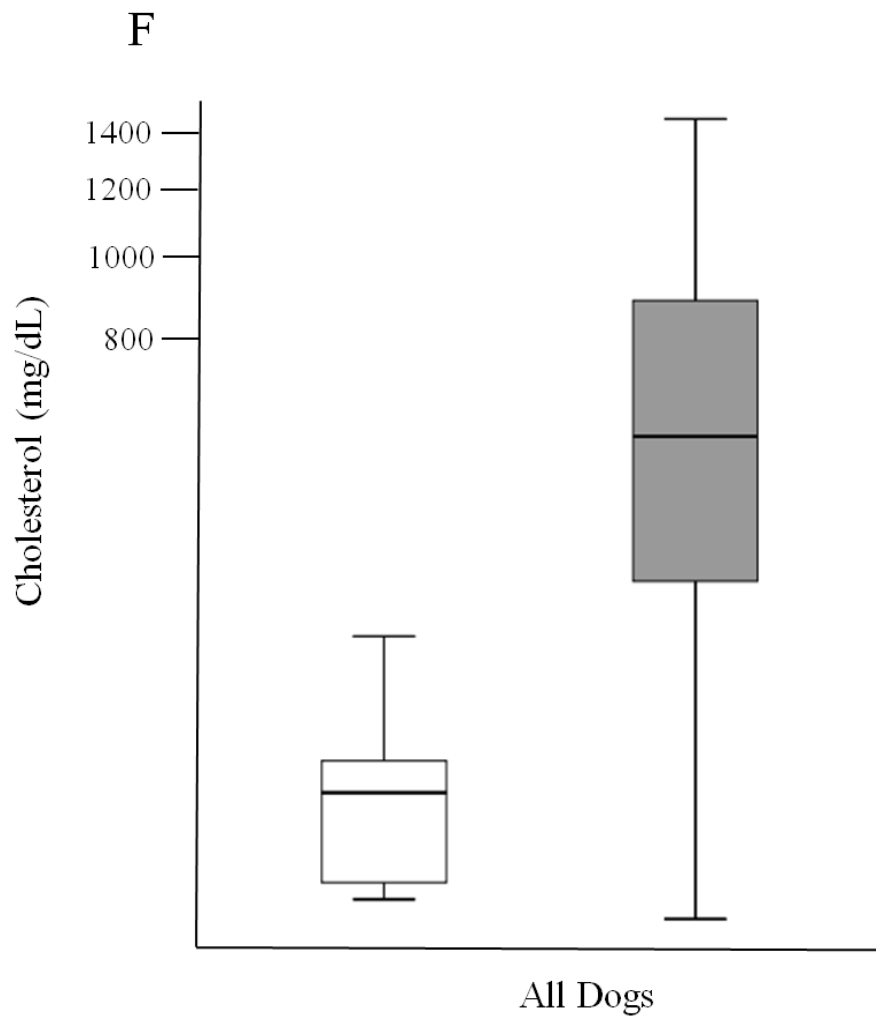


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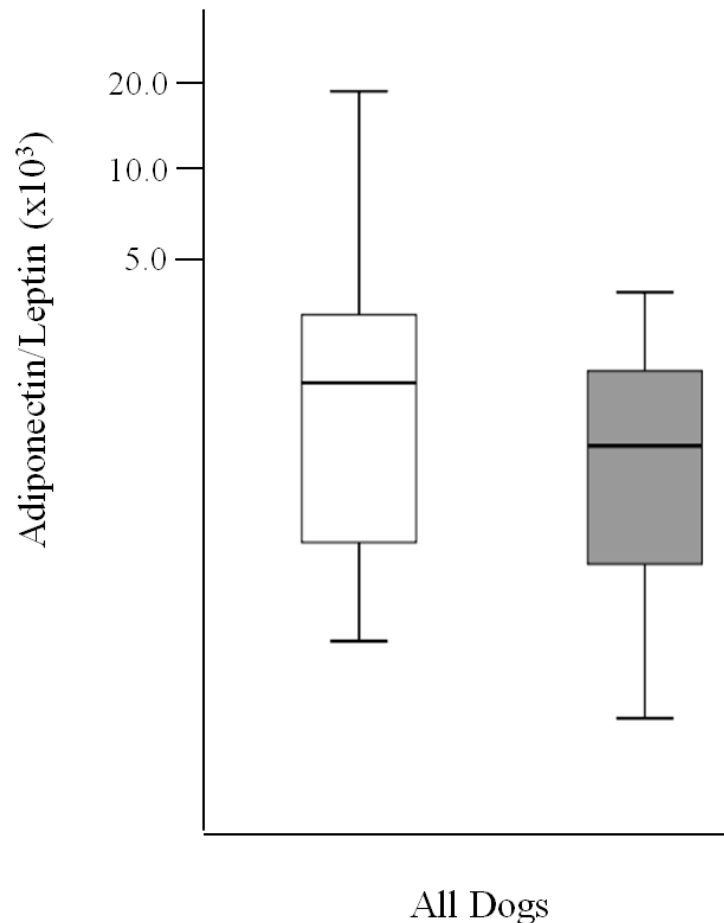


Adiponectin to leptin ratio in healthy and hypothyroid dogs

The ratio of serum concentrations of adiponectin to serum concentration of leptin (Mean; 95% CI) was not significantly different between healthy (1.41×10^3 ; 0.55×10^3 , 2.44×10^3) and hypothyroid (0.97×10^3 ; 0.54×10^3 , 1.73×10^3) dogs (Figure 16).

Figure 16. Adiponectin to leptin ratio in healthy (□) and hypothyroid dogs (■).†

† The ration is presented in a natural logarithmic scale. The horizontal bar within the box plot indicates the median, the box contains the middle half of the results, the T-bars indicate the range.



Associations among BCS and serum concentrations of hormones, lipids, and glucose in healthy and hypothyroid dogs

Serum concentrations of leptin were significantly correlated to BCS both in healthy ($r = 0.73$, $P < 0.001$) and hypothyroid dogs ($r = 0.57$, $P = 0.003$).

Serum concentrations of adiponectin were significantly correlated to concentrations of triglyceride ($r = -0.52$; $P = 0.026$) in the healthy dogs group, and to concentrations of cholesterol

($r = 0.60$, $P = 0.009$) in the hypothyroid dogs group. There was a nearly significant correlation between adiponectin and triglyceride in the hypothyroid group ($r = 0.46$, $P = 0.058$).

Serum concentrations of insulin were significantly correlated to concentrations of triglyceride ($r = 0.73$; $P < 0.001$) and cholesterol ($r = 0.62$; $P = 0.001$) in hypothyroid dogs but the healthy dogs group.

Associations between hormones, and, biochemical parameters or total T4 among hypothyroid dogs

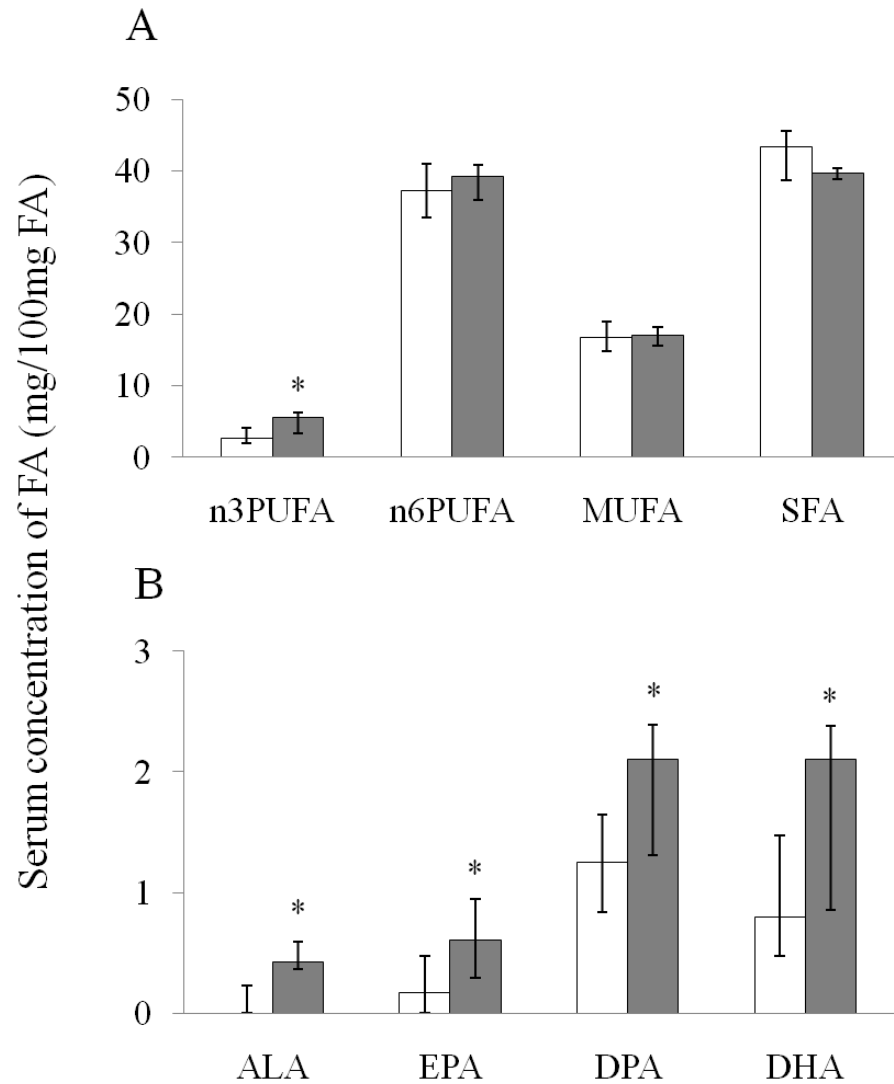
Serum concentrations of leptin and of adiponectin were significantly correlated to total T4 ($r = -0.43$, $P = 0.033$ and $r = -0.57$, $P = 0.014$, respectively). Serum concentrations of insulin were significantly correlated to fold increase in ALT activity ($r = 0.68$, $P = 0.031$). Serum concentrations of triglyceride and cholesterol were significantly correlated to fold increase in ALT activity ($r = 0.53$, $P = 0.016$ and $r = 0.50$, $P = 0.019$) and concentrations of total T4 ($r = -0.56$, $P = 0.008$ and $r = -0.69$, $P < 0.001$). Concentrations of triglyceride were significantly correlated to concentrations of cholesterol ($r = 0.72$, $P < 0.001$).

Serum concentrations of FAs in healthy dogs and dogs with hypothyroidism

Serum FA analysis was available for 10 dogs with hypothyroidism and 11 healthy dogs. Dogs with hypothyroidism had significantly higher serum concentrations of n3PUFAs as well as higher concentrations of each of the individual n3PUFAs (Figure 17 A and B).

Figure 17. Serum concentration of total FAs within categories (A) and individual n3PUFAs (B) in healthy dogs (□) and dogs with hypothyroidism (■).†

† Bar represents the median and error bars represent interquartile range. * $P < 0.01$ compared to healthy dogs.



Associations between concentrations of total or individual n3PUFAs and concentrations of hormones, lipids, and glucose in dogs with hypothyroidism

No significant associations were present between total n3PUFAs or any individual n3PUFAs and any of the outcome variables.

Associations between concentrations of total or individual n3PUFAs and biochemical parameters in dogs with hypothyroidism

Serum concentrations of EPA were significantly correlated to fold increases in ALT and AST activities ($r = 0.64$, $P = 0.048$ and $r = 0.65$, $P = 0.043$, respectively). Serum concentrations of DHA were significantly correlated to fold increase in ALT activity ($r = 0.67$, $P = 0.033$) and nearly significant to fold increase in AST activity ($r = 0.62$, $P = 0.054$).

2.2.3. Circulating concentrations of adipokines and n3PUFAs in feline diabetes mellitus

Study aims

- To determine the effect of feline DM on circulating concentrations of adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol by comparing serum concentrations among cats diagnosed with UDM, cats diagnosed with DKA, and healthy cats.
- To determine the effect of n3PUFAs on circulating concentrations of adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol in feline DM.
- To compare serum concentrations of n3PUFAs between healthy cats and cats with DM.

Methods

Study design

Eleven cats with DM and 55 healthy adult cats were enrolled in the study. Client-owned cats with newly-diagnosed DM were recruited from the Veterinary Teaching Hospitals at Michigan State University (MSU) and at the Hebrew University of Jerusalem (HUJI) and were allocated into 1 of 2 groups: 1) uncomplicated DM (UDM; n=5), and 2) diabetic ketoacidosis (DKA; n=6). Diagnosis of DM was based on clinical signs and clinical pathology including persistent fasting hyperglycemia and glucosuria. Diagnosis of DKA was based on presence of additional ketonuria and/or serum concentrations of β -hydroxybutiric acid. Exclusion criteria included administration of insulin, oral hypoglycemic drugs, or glucocorticoids prior to sample collection. The healthy cats were client-owned cats visiting MSU for routine check-up. The cats were considered to be healthy based on physical examination and routine biochemistry analysis. Additional inclusion criteria were based on medical and dietary history for a 3 month period preceding sample collection and included: 1) absence of any clinical signs indicating illness; 2) exclusive feeding of a commercially available, nutritionally balanced and complete adult cat diet, and; 3) no administration of dietary supplements or medications.

Body condition of all cats was evaluated by 2 individuals using BCS system on a 1- 9 scale (Laflamme, 1997a) and fasted serum samples were collected. The study was performed in compliance with Michigan State University and the Hebrew University of Jerusalem guidelines for research in animals. Informed consent was obtained from all owners.

Serum analysis

Serum was separated immediately following blood collection and frozen at -80 °C until analyzed. Serum adiponectin, leptin and insulin concentrations were measured using commercially available assays previously used in cats (Backus et al., 2000; Hoenig et al., 2007) and validated in our laboratory (Mouse/rat Adiponectin ELISA kit-B-Bridge, Multispecies Leptin RIA kit- Millipore, Human Insulin RIA kit- Diagnostic Systems Laboratories- (See Appendix C, Protocol 1 and 2). Serum concentrations of glucose, cholesterol and triglyceride were measured using a spectrophotometric method (Kodak Ektachem DT60 II Clinical Products Division, Eastman Kodak Co). Additional biochemistry parameters were measured using automated chemistry analyzers (AU640^e Chemistry-Immuno System, Olympus America- at MSU, Cobas Mira Chemistry Automatic Analyser, Roche- at HUJI). Serum FA analysis was performed by gas chromatography (See Appendix C, Protocol 3).

Data analysis

Associations between diabetic disease and the outcome variables (serum concentrations of adiponectin, leptin, insulin, glucose, triglyceride, or cholesterol) were evaluated using GLM including group (healthy cats, cats with UDM, and cats with DKA) as a factor, and BCS, age, gender, and location (MSU or HUJI) as covariates. The additional covariates were included to control for potential confounding of the relationship between disease and the outcome variables. The link function and the linear predictor for each of the variables are of the form $E[Y|x] = a + bx$. Y is a natural logarithmic transformation of the measured values and has a normal distribution (as confirmed by P-P plots) with a mean ($a + bx$) and constant variances. All interaction effects between group and the additional covariates were assessed. Covariates and interaction terms with significance level of effect $P \geq 0.1$ were removed from the models.

Association between diabetic disease and adiponectin to leptin ratio was determined using GLM with group as a factor. Bonferroni adjustment was applied for multiple posthoc pairwise comparisons among the groups.

Model fitting and post post-hoc categorization- The covariate location was removed from the models evaluating the associations between disease and all outcome variables. The covariate gender was included in all models but those for triglyceride and cholesterol. The covariate age was included in the model for insulin. No significant interaction effects were present between BCS and group in the analyses of the association of disease and any of the outcome variables.

When means of the outcome variable in the groups of cats with DM (UDM and DKA) were all either higher or lower compared to healthy cats in the analyses described above, further analyses of the associations between DM and the outcome variable were performed using all cats with DM grouped together and healthy cats as the group factor.

Linear correlations between the outcome variables and BCS were evaluated using Spearman correlation coefficient. Linear correlations between the outcome variables and fold increases in serum concentrations of total bilirubin, albumin, creatinine, and blood urea nitrogen, or fold increases of serum activities of ALT, AST, and ALP were evaluated using Pearson correlation coefficient. Natural log transformation was used for any non-normally distributed variable (all variables but total bilirubin).

Associations between serum concentrations of n3PUFAs or serum concentrations of individual n3PUFAs (EPA, DPA, DHA, and ALA) and serum concentrations of adiponectin, leptin, insulin, glucose, triglyceride, or cholesterol within the group of cats with DM were evaluated using GLM including n3PUFAs or individual n3PUFAs, BCS, gender, and age as covariates. Absence of potential colinearity among serum concentrations of EPA, DPA, DHA,

and ALA was confirmed. The additional covariates (BCS, gender, and age) were included to control for potential confounding of the relationship between n3PUFAs and the outcome variables (adiponectin, leptin, insulin, glucose, triglyceride, or cholesterol). The link function and the linear predictor for each of the variables are of the form $E[Y|x] = a + bx$. Y is a natural logarithmic transformation of the measured values and has a normal distribution (as confirmed by P–P plots) with a mean ($a + bx$) and constant variances. All interaction effects between n3PUFAs and other variables in the models were assessed.

Model fitting and post-hoc categorization- The covariates age and gender were removed from all models due to insignificant effect ($P \geq 1.0$). No significant interaction effects were present between BCS and group.

Linear correlations between serum concentrations of total or individual n3PUFAs and serum activities of liver enzymes or concentration of bilirubin within cats with DM were evaluated using Spearman correlation coefficient.

Mann-Whitney U test was used to compare serum concentrations of FAs between healthy cats and cats with DM.

Data were analyzed using a commercially available statistic program (SPSS 19.0 for Windows). $P < 0.05$ was considered statistically significant.

Results

Cats

The healthy cats group included was the same as in study 1.2.3. The cats with DM included 3 neutered females, 4 neutered males, and 4 intact males. Age ranged from 3 to 15

years (mean: 9.3 y). BCS ranged from 2 to 9 (Median: 5). Breeds consisted of domestic long hair (4), domestic short hair (2), domestic medium hair (1), Persian (1), and Siamese (1). One cat had concurrent pancreatitis. Changes in biochemistry parameters in cats with DM are presented in Table 25.

Table 25. Biochemical changes in cats with DM.*

Variable	UDM	DKA
Blood urea nitrogen	0.6 (0.3, 1.0)	1.7 (0.6, 3.0)
Creatinine	0.8 (0.4, 0.9)	1.2 (0.5, 2.3)
Albumin	0.7 (0.4, 0.9)	0.7 (0.5,0.8)
Total bilirubin	1.4 (0.8, 2.7)	7.3 (1.6, 14.9)
Alkaline phosphatase	1.6 (1.1, 2.9)	0.9 (0.8, 4.4)
Alanine aminotransferase	1.3 (0.3, 2.4)	1.6 (0.8, 2.3)
Aspartate aminotransferase	1.2 (0.8, 3.6)	2.9 (1.5, 4.6)

* Values presented are median (range) of fold increase of the upper reference range value.

Associations between diabetic disease and serum concentrations of hormones, lipids, and glucose

Adjusted serum concentrations of hormones, lipids, and glucose in healthy cats, cats with UDM, and cats with DKA are presented in Table 26 and the measured values of serum concentrations are presents in Figure 18.

Serum concentrations of leptin were significantly higher both in cats with UDM and in cats with DKA than in healthy cats; and cats with DKA had significantly higher concentrations than cats with UDM. Serum concentrations of insulin were significantly higher in cats with

UDM but not in cats with DKA compared to healthy cats, while concentrations of glucose, triglyceride, and cholesterol were significantly higher both in cats with UDM and in cats with DKA compared to healthy cats (Table 26).

When all cats with DM were grouped together, serum concentrations of leptin were significantly ($P < 0.001$) higher in cats with DM (10.8 ng/mL; 8.8, 13.4) than in healthy cats (3.6 ng/mL; 3.1, 4.3), following adjustment for gender and BCS; serum concentrations of insulin were significantly ($P = 0.019$) higher in cats with DM (45 pmol/L; 29, 70) than in healthy cats (23 pmol/L; 17, 31), following adjustment for age, gender and BCS; serum concentrations of glucose were significantly ($P < 0.001$) higher in cats with DM (455 mg/dL; 393, 527) than in healthy cats (91 mg/dL; 82, 102), following adjustment for gender and BCS; serum concentrations of triglyceride were significantly ($P < 0.001$) higher in cats with DM (152 mg/dL; 115, 201) than in healthy cats (76 mg/dL; 67, 85), following adjustment for BCS; and serum concentrations of cholesterol were significantly ($P < 0.001$) higher in cats with DM (216 mg/dL; 178, 261) than in healthy cats (135 mg/dL; 125, 147), following adjustment for BCS. No significant difference in concentrations of adiponectin was present between healthy cats and cats with DM.

Table 26. Serum concentrations of hormones, glucose, and lipids in cats with DM and healthy cats.*

Variable	Covariates†	Group		
		Healthy‡	UDM‡	DKA‡
Adiponectin (µg/mL)	BCS, gender	1.7 (1.1, 2.6)	1.6 (0.7, 3.5)	1.2 (0.6, 2.6)
Leptin (ng/mL)	BCS, gender	3.7 (3.2, 4.3) ^a	7.4 (5.6, 9.7) ^a	15.4 (5.6, 20.0) ^a
Insulin (pmol/L)	BCS, gender, age	22 (16, 29) ^b	72 (40, 129) ^b	30 (17, 52)
Glucose (mg/dL)	BCS, gender	96 (86, 106) ^{a,d}	364 (301, 441) ^d	465 (388, 558) ^a
Triglyceride (mg/dL)	BCS	76 (67, 85) ^{a,c}	131 (88, 195) ^c	173 (119, 253) ^a
Cholesterol (mg/dL)	BCS	135 (125, 147) ^{a,c}	197 (150, 259) ^c	235 (182, 305) ^a

* Associations between group and serum concentrations of hormones, glucose, or lipids were determined using GLM. †Covariates BCS, age, gender, and location as well as interaction terms between group and each of the covariates were evaluated in each model. Covariates with an effect of significance level $P \leq 0.1$ were included in each model. ‡ Adjusted mean (95% CI); Same letter within a row indicates a significant difference with Bonferroni correction for multiple comparisons: a,d- $P < 0.001$; b- $P < 0.005$; c- $P < 0.05$.

Figure 18. Serum concentrations of adiponectin (A), leptin (B), insulin (C), glucose (D), triglyceride (E), and cholesterol (F) and in healthy cats (□), cats with UDM (▒), and cats with DKA (■).†

† Serum concentrations are presented on a natural logarithmic scale of the measured concentrations. The horizontal bar within the box plot indicates the median, the box contains the middle half of the results, the T-bars indicate the range, and the circles and stars indicate outlying and extreme data.

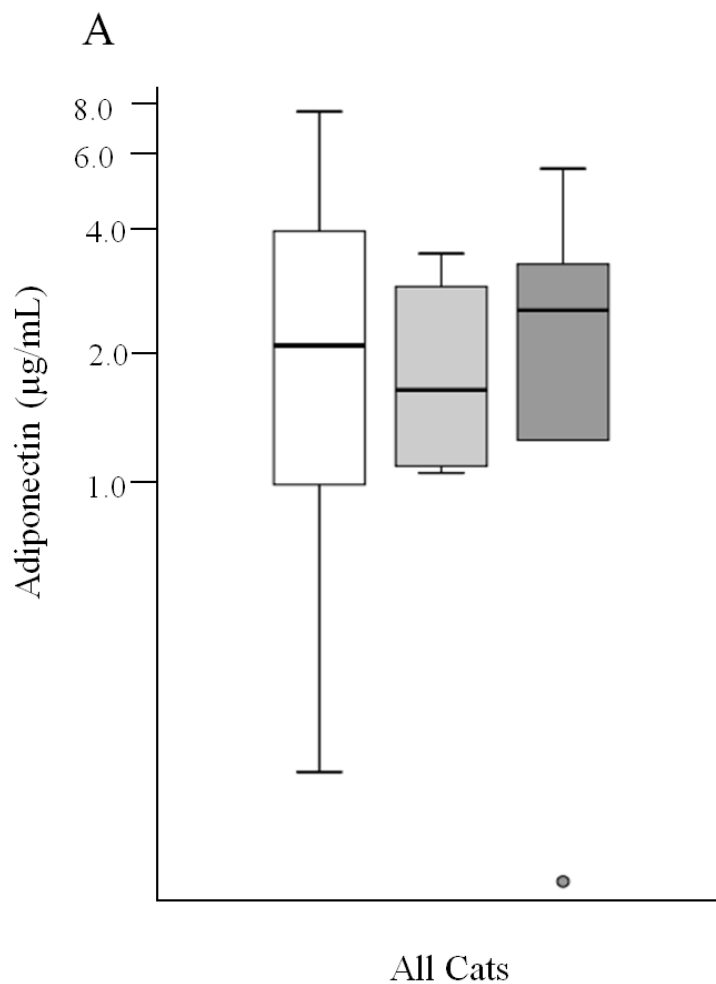


Figure 18 (cont'd)

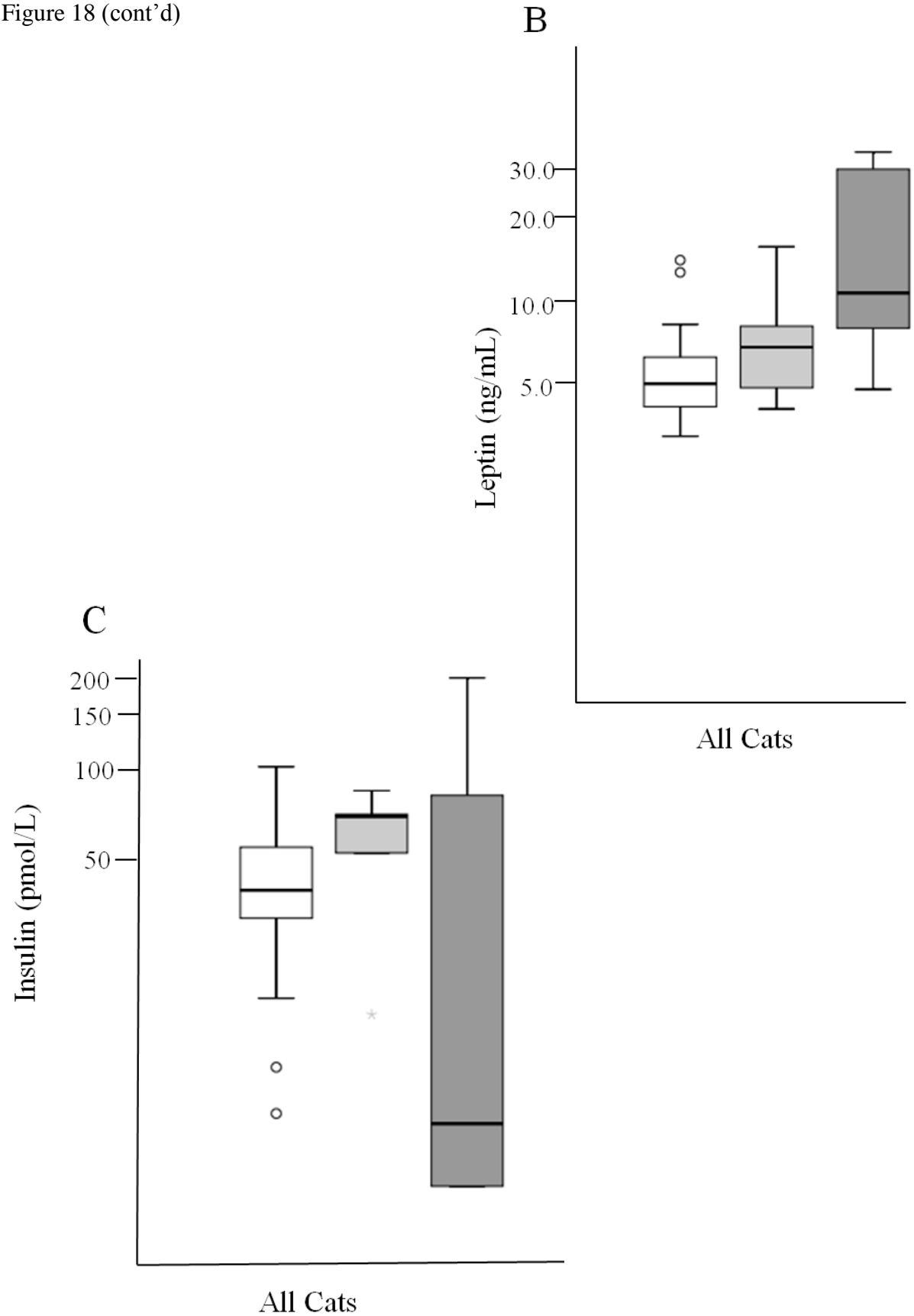


Figure 18 (cont'd)

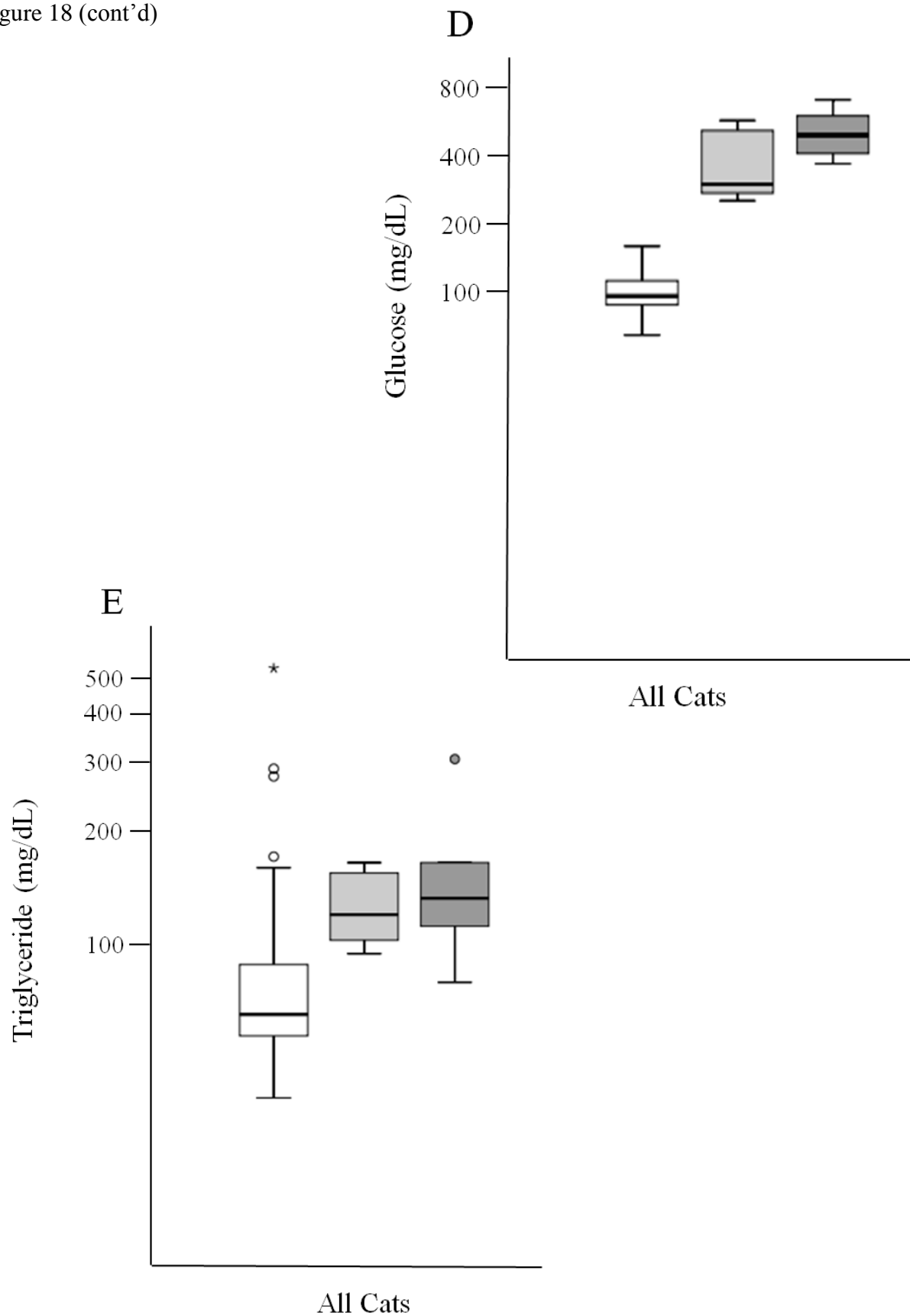
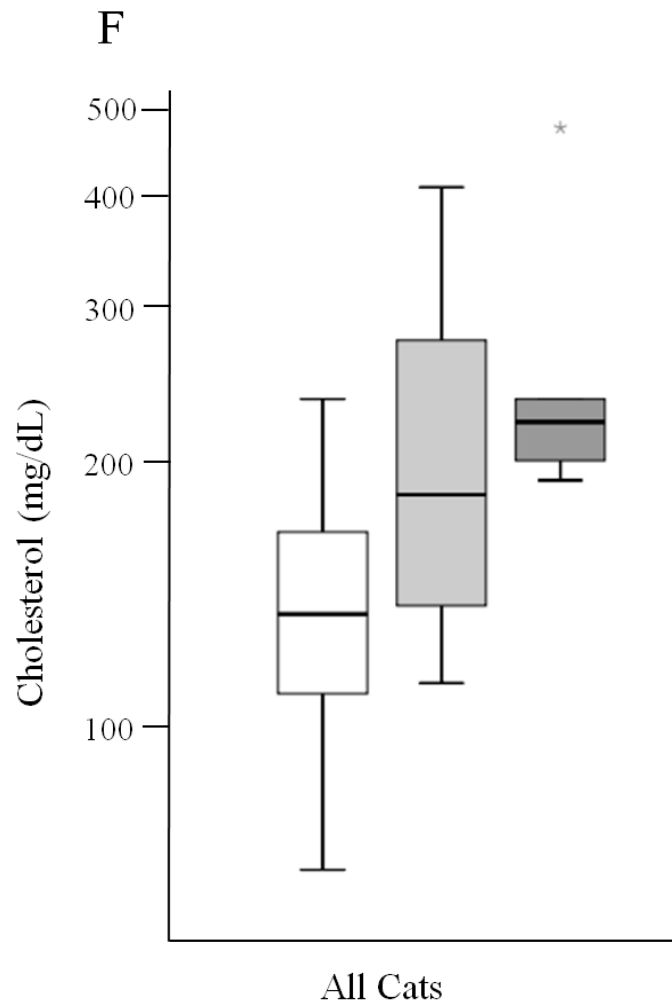


Figure 18 (cont'd)

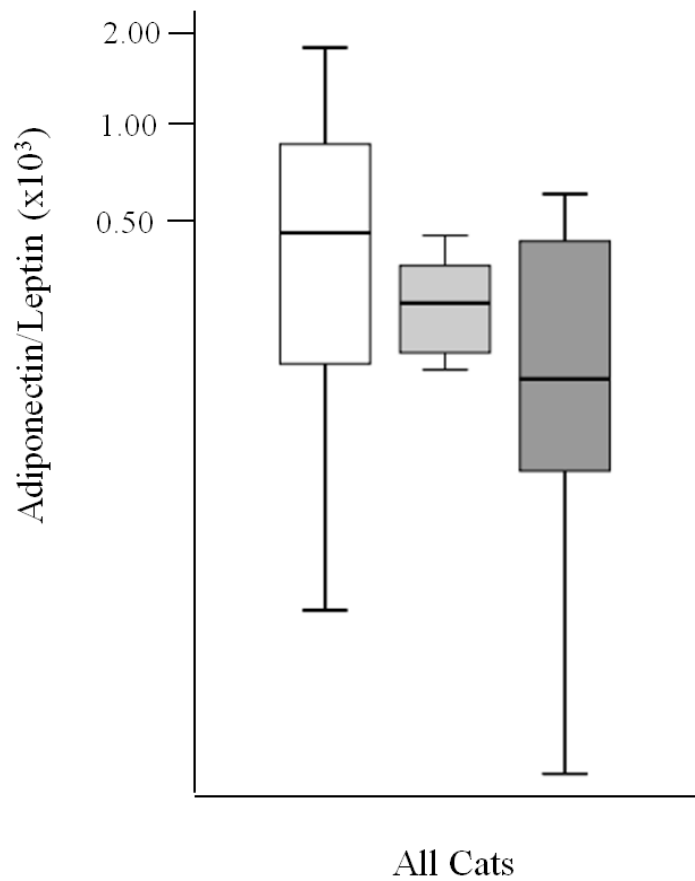


Adiponectin to leptin ratio in cats with DM

The ratio of serum concentrations of adiponectin to serum concentration of leptin (Mean; 95% CI) was significantly ($P = 0.047$) lower in cats with DM (0.18×10^3 ; 0.09×10^3 , 0.35×10^3) compared to healthy cats (0.37×10^3 ; 0.28×10^3 , 0.50×10^3) when all cats with diabetes were grouped together, but no significant difference was present between cats with UDM and cats with DKA (Figure 19).

Figure 19. Adiponectin to leptin ratio in healthy cats (□), cats with UDM (▒), and cats with DKA (■).†

† The ratio is presented on an natural logarithmic scale. The horizontal bar within the box plot indicates the median, the box contains the middle half of the results, and the T-bars indicate the range.



Associations among BCS and serum concentrations of hormones, lipids, and glucose in cats with DM

When all cats with DM were grouped together, serum concentrations of adiponectin were significantly negatively correlated to fold increase in serum triglyceride concentration ($r = -0.76$, $P = 0.007$). No significant linear correlations between BCS and any of the outcome variables were revealed.

Associations between hormones, glucose, lipids, and biochemical parameters among cats with DM

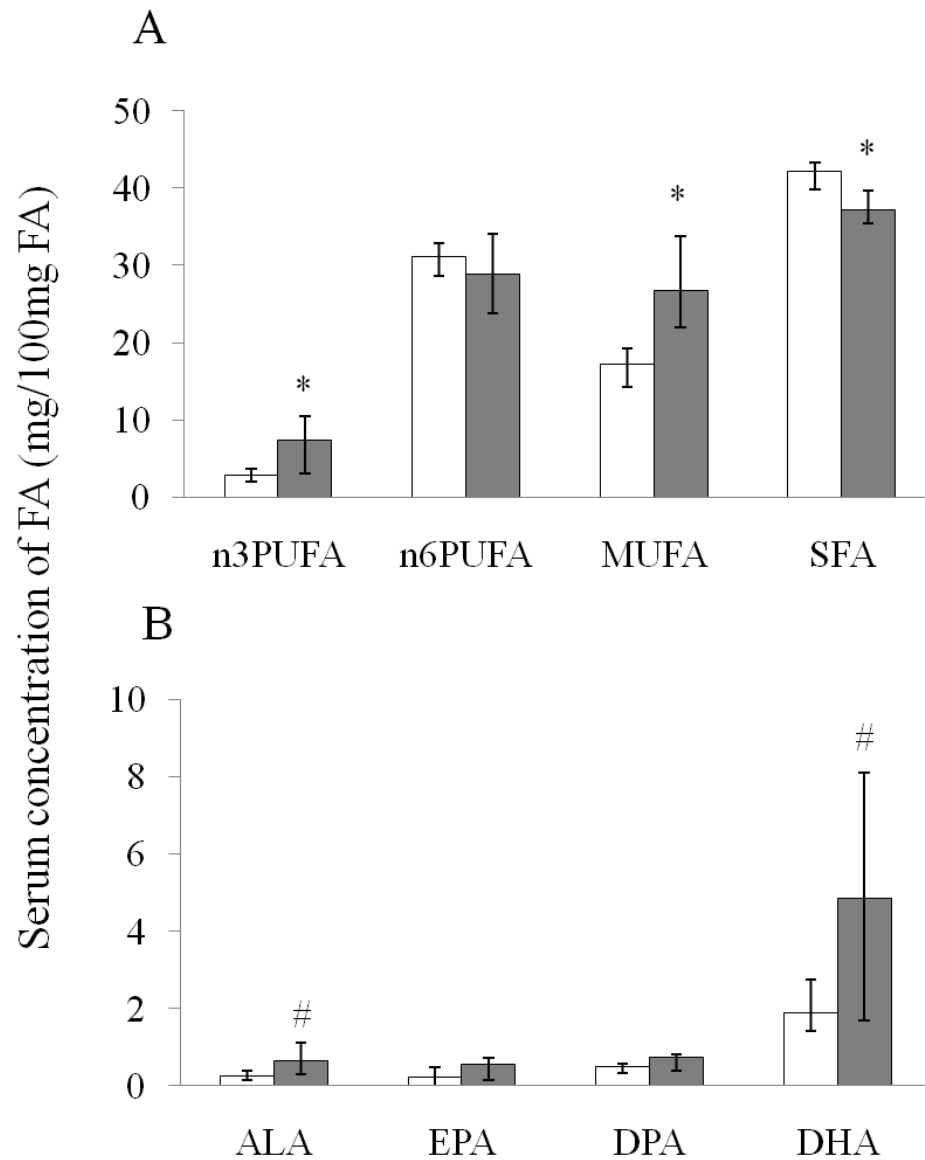
Serum concentrations of leptin were significantly correlated to concentrations of BUN ($r = 0.75$, $P = 0.033$) and creatinine ($r = 0.88$, $P < 0.001$).

Serum concentrations of FAs in healthy cats and cats with DM

Serum FA analysis was available for 7 cats with DM (5 non-obese and 2 obese) and all healthy cats. Cats with DM had significantly higher serum concentrations of n3PUFAs and MUFAs and significantly lower concentrations of SFAs than healthy cats. Of the individual n3PUFAs, serum concentrations of ALA and DHA were significantly higher in cats with DM (Figure 20 A and B). No significant differences in serum concentrations of FAs were present between non-obese and obese cats in the healthy or DM groups.

Figure 20. Serum concentration of total FAs within categories (A) and individual n3PUFAs (B) in healthy cats (□) and cats with DM (■).†

† Bar represents the median and error bars represent interquartile range * $P < 0.001$, # $P < 0.05$ compared to healthy cats.



Associations between concentrations of total or individual n3PUFAs and serum concentrations of hormones, lipids, and glucose in cats with DM

An increase of 1 mg/100mg FA in serum concentration of total n3PUFAs was associated with a 15% increase in adjusted geometric mean of serum concentration of glucose (95% CI of fold increase: 1.09, 1.20; Partial $\eta^2 = 95\%$; $P = 0.001$). No other significant associations were revealed between the outcome variables and total n3PUFAs or any of the individual n3PUFAs.

Associations between concentrations of total or individual n3PUFAs and biochemical parameters in cats with DM

Concentrations of EPA were significantly correlated to fold increase in ALP activity ($r = -0.90$, $P = 0.037$) and concentrations of ALA were significantly correlated to fold increase in bilirubin concentrations ($r = -0.99$, $P < 0.001$).

2.2.4. Circulating concentrations of adipokines and n3PUFAs in canine diabetes mellitus

Study aims

- To determine the effect of canine DM on circulating concentrations of adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol by comparing serum concentrations among dogs diagnosed with UDM, dogs diagnosed DKA, and healthy dogs.
- To determine the effect of n3PUFAs on circulating concentrations of adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol in canine DM.
- To compare serum concentrations of n3PUFAs between healthy dogs and dogs with DM.

Methods

Study design

Ten dogs with DM and 18 healthy adult dogs were enrolled in the study. Client-owned dogs with newly-diagnosed DM were recruited from the Hebrew University of Jerusalem (HUJI) and were allocated into 1 of 2 groups: 1) UDM (n=4), and 2) DKA (n=6). Diagnosis of DM was based on clinical signs and clinical pathology including persistent fasting hyperglycemia and glucosuria. Diagnosis of DKA was based on presence of additional ketonuria and/or serum concentrations of β -hydroxybutiric acid. Exclusion criteria included administration of insulin, oral hypoglycemic drugs, or glucocorticoids prior to sample collection. The healthy dogs were client-owned dogs visiting HUJI for routine check-ups. The dogs were considered to be healthy based on physical examination and routine biochemistry analysis. Additional inclusion criteria were based on medical and dietary history for a 3 month period preceding sample collection and included: 1) absence of any clinical signs indicating illness; 2) exclusive feeding of a commercially available, nutritionally balanced and complete adult cat diet, and; 3) no administration of dietary supplements or medications.

Body condition of all dogs was evaluated by 2 individuals using BCS system on a scale of 1 to 9 (Laflamme, 1997b) and fasted serum samples were collected. The study was performed in compliance with Michigan State University and the Hebrew University of Jerusalem guidelines for research in animals. Informed consent was obtained from all owners.

Serum analysis

Serum was separated immediately following blood collection and frozen at -80 °C until analyzed. Serum adiponectin, leptin and insulin concentrations were measured using commercially available assays (Canine Adiponectin ELISA, Millipore; Canine Leptin ELISA, Millipore; Human Insulin RIA, Diagnostic Systems Laboratories- See Appendix C, Protocol 1 and 2). Serum glucose concentrations were measured using an automated chemistry analyzer (AU640^e Chemistry-Immuno System, Olympus America) and concentrations of triglyceride were measured using a spectrophotometric method (Kodak Ektachem DT60 II Clinical Products Division, Eastman Kodak Co). Serum total lipid content was determined and FA analysis was performed by gas chromatography (See Appendix C, Protocol 3).

Data analysis

Associations between diabetic disease and the outcome variables (serum concentrations of adiponectin, leptin, insulin, glucose, triglyceride, or cholesterol) were evaluated using GLM including group (healthy dogs, dogs with UDM, and dogs with DKA) as a factor, and BCS, age and gender as covariates. The additional covariates were included to control for potential confounding of the relationship between disease and the outcome variables. The link function and the linear predictor for each of the variables are of the form $E[Y|x] = a + bx$. Y is a natural logarithmic transformation of the measured values and has a normal distribution (as confirmed by P-P plots) with a mean ($a + bx$) and constant variances. All interaction effects between group and the additional covariates were assessed. Covariates and interaction terms with significance level of effect $P \geq 0.1$ were removed from the models. Association between diabetic disease and

adiponectin to leptin ratio was determined using GLM with group as a factor. Bonferroni adjustment was applied for multiple posthoc pairwise comparisons among the groups.

Model fitting and post-hoc categorization - All additional covariates were removed from all models but BCS in the models for analyses of adiponectin, leptin, and insulin. No significant interaction effects were present between BCS and group in the analyses of the association of disease and any of the outcome variables.

When means of the outcome variable in the groups of dogs with DM (UDM and DKA) were all either higher or lower compared to healthy dogs in the analyses described above, further analyses of the associations between DM and the outcome variable were performed using all dogs with DM grouped together and healthy dogs as the group factor.

Linear correlations between the outcome variables and BCS were evaluated using Spearman correlation coefficient. Linear correlations between the outcome variables and fold increases in serum concentrations of total bilirubin or albumin, or fold increases in serum activities of ALT, AST, and ALP were evaluated using Pearson correlation coefficient. Natural log transformation was used for any non-normally distributed variable (all variables but total bilirubin).

Associations between serum concentrations of n3PUFAs or serum concentrations of individual n3PUFAs (EPA, DPA, DHA, and ALA) and serum concentrations of adiponectin, leptin, insulin, glucose, triglyceride, or cholesterol within the group of dogs with DM were evaluated using GLM including n3PUFAs or individual n3PUFAs and BCS as covariates. Each n3PUFA was evaluated in a separate model due to the small sample size. The additional covariate (BCS) was included to control for potential confounding of the relationship between n3PUFAs and the outcome variables (adiponectin, leptin, insulin, glucose, triglyceride, or

cholesterol). The link function and the linear predictor for each of the variables are of the form $E[Y|x] = a + bx$. Y is a natural logarithmic transformation of the measured values and has a normal distribution (as confirmed by P–P plots) with a mean ($a + bx$) and constant variances. All interaction effects between n3PUFAs and BCS were assessed.

Model fitting and post-hoc categorization – No significant interaction effects between group and BCS were present. The covariate BCS was removed from all models but the models for analyses of leptin and insulin.

Linear correlations between serum concentrations of total or individual n3PUFAs and serum activities of liver enzymes or concentration of bilirubin within the group of dogs with DM were evaluated using Spearman correlation coefficient.

Mann-Whitney U test was used to compare serum concentrations of FAs between healthy dogs and dogs with DM.

Data were analyzed using a commercially available statistic program (SPSS 19.0 for Windows). $P < 0.05$ was considered statistically significant.

Results

Dogs

The healthy dogs group consisted of the dogs included in study 2.2.2. The dogs with DM included 5 intact females, 3 intact males, 1 neutered female, and 1 neutered male. Age ranged from 7 to 11 years (mean: 8.6 y). BCS ranged from 4 to 9 (Median: 6). Breeds consisted of Miniature Pinscher (3), Miniature Poodle (2), Siberian Husky (1), Keeshond (1), Great Dane (1),

Doberman Pinscher (1), and mixed breed (1). Changes in biochemistry parameters in dogs with DM are presented in Table 27.

Table 27. Biochemical changes in dogs with DM.*

Variable	UDM	DKA
Blood urea nitrogen	0.4 (0.4, 0.8)	0.5 (0.2, 2.1)
Creatinine	0.6 (0.3, 0.7)	0.2 (0.0, 1.6)
Albumin	0.9 (0.8, 0.9)	0.8 (0.6, 1.0)
Total bilirubin	0.5 (0.5, 2.3)	2.2 (0.5, 3.9)
Alkaline phosphatase	1.8 (1.5, 13.4)	8.4 (2.6, 74.9)
Alanine aminotransferase	0.7 (0.3, 2.2)	1.8 (0.3, 2.0)
Aspartate aminotransferase	0.9 (0.6, 4.5)	2.5 (0.7, 5.5)

* Values presented are median (range) of fold increases of measured values of serum concentrations compared to the upper end of the reference range.

Associations among BCS and serum concentrations of hormones, lipids, and glucose in dogs with DM

No significant linear correlations were revealed.

Serum concentrations of hormones, lipids, and glucose in healthy dogs and dogs with DM

Adjusted serum concentrations of hormones, lipids, and glucose among healthy dogs, dogs with UDM, and dogs with DKA are presented in Table 28 and the measured values are presents in Figure 21.

Serum concentrations of leptin were significantly lower in dogs with UDM than in healthy dogs, but not different between dogs with DKA and healthy dogs. Serum concentrations of insulin were significantly lower and concentrations of glucose were significantly higher both in dogs with UDM and dogs with DKA compared to healthy dogs. Serum concentrations of triglyceride and cholesterol were significantly higher in dogs with DKA compared to healthy dogs (Table 28).

When all dogs with DM were grouped together, serum concentrations of insulin were significantly ($P = 0.009$) lower in dogs with DM (40 pmol/L; 21, 80) than in healthy dogs (128 pmol/L; 78, 210), serum concentrations of glucose were significantly ($P < 0.001$) higher in dogs with DM (366 mg/dL; 267, 501) than in healthy dogs (92 mg/dL; 72, 118), and serum concentrations of cholesterol were significantly ($P = 0.001$) higher in dogs with DM (328 mg/dL; 267, 403) than in healthy dogs (209 mg/dL; 186, 234).

Table 28. Serum concentrations of hormones, glucose, and lipids in dogs with DM and healthy dogs.*

Variable	Covariates†	Group		
		Healthy‡	UDM‡	DKA‡
Adiponectin (µg/mL)	BCS	8.9 (6.4, 12.5)	9.7 (4.8, 19.7)	6.7 (3.3, 13.7)
Leptin (ng/mL)	BCS	5.7 (3.6, 9.2) ^a	0.83 (0.27, 2.5) ^a	5.6 (1.8, 16.9)
Insulin (pmol/L)	BCS	128 (87, 188) ^{a,b}	13 (5, 33) ^b	36 (16, 80) ^a
Glucose (mg/dL)	-	92 (54, 126) ^{b,c}	382 (306, 478) ^b	503 (430, 590) ^c
Triglyceride (mg/dL)	-	82 (67, 85) ^b	63 (18, 224)	684 (305, 1531) ^b
Cholesterol (mg/dL)	-	206 (182, 232) ^b	302 (224, 406)	440 (340, 570) ^b

* Associations between group and serum concentrations of hormones, glucose, or lipids were determined using GLM. † Covariates BCS, age, gender, and location as well as interaction terms between group and each of the covariates were evaluated in each model. Covariates with an effect of significance level $P \leq 0.1$ were included in each model. ‡ Adjusted mean (95% CI); Same letter within a row indicates a significant difference with Bonferroni correction for multiple comparisons: a, c- $P < 0.05$; b- $P < 0.001$.

Figure 21. Serum concentrations of adiponectin (A), leptin (B), insulin (C), glucose (D), triglyceride (E), and cholesterol (F) and in healthy dogs (□), dogs with UDM (▨), and dogs with DKA (■).†

† Serum concentrations are presented on a natural logarithmic scale of the measured concentrations. The horizontal bar within the box plot indicates the median, the box contains the middle half of the results, the T-bars indicate the range, and the circles indicate outlying data.

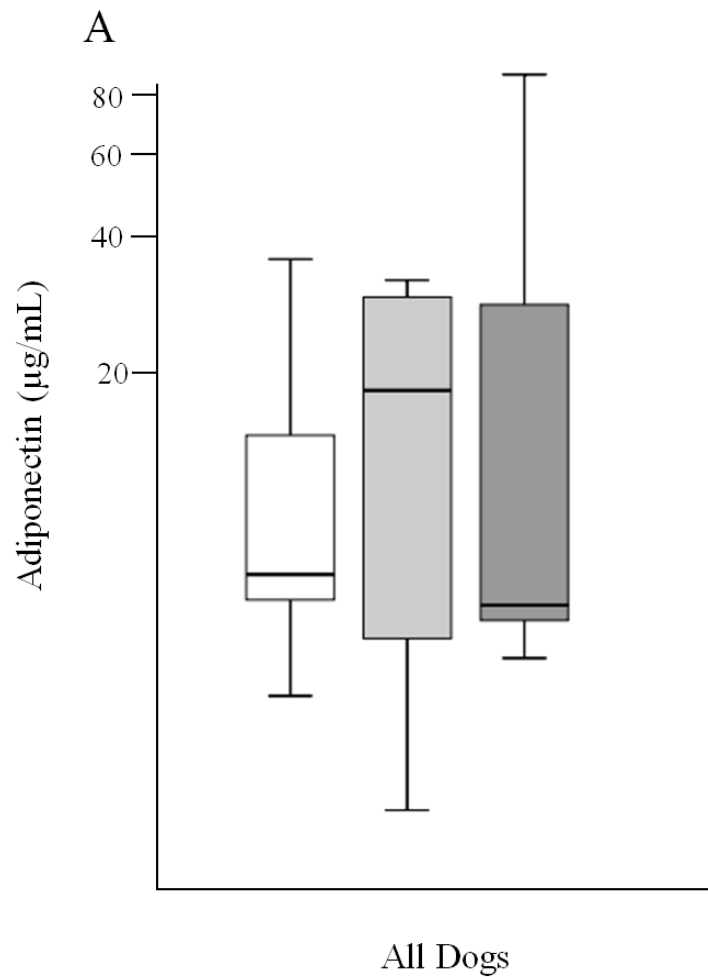


Figure 21 (cont'd)

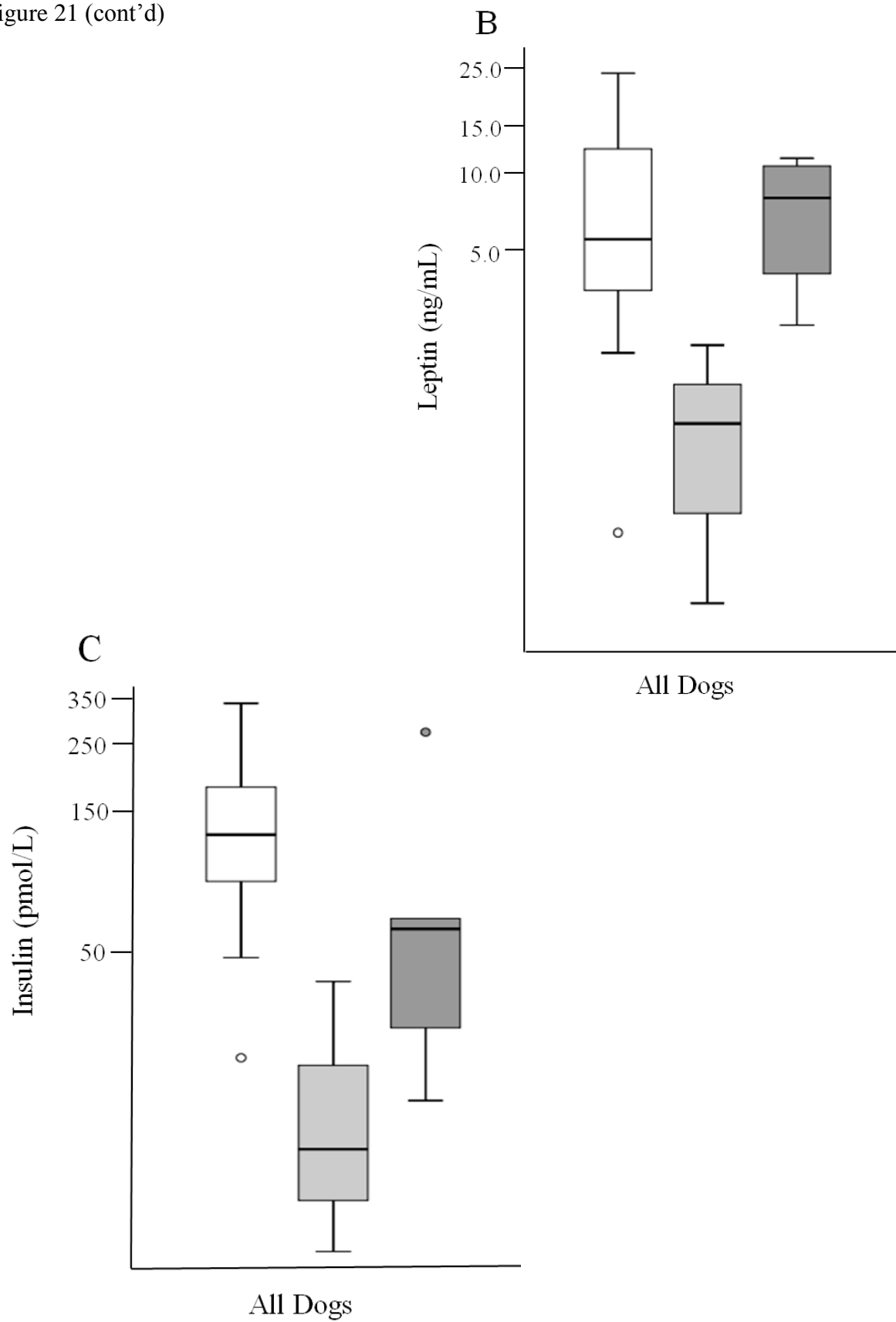


Figure 21 (cont'd)

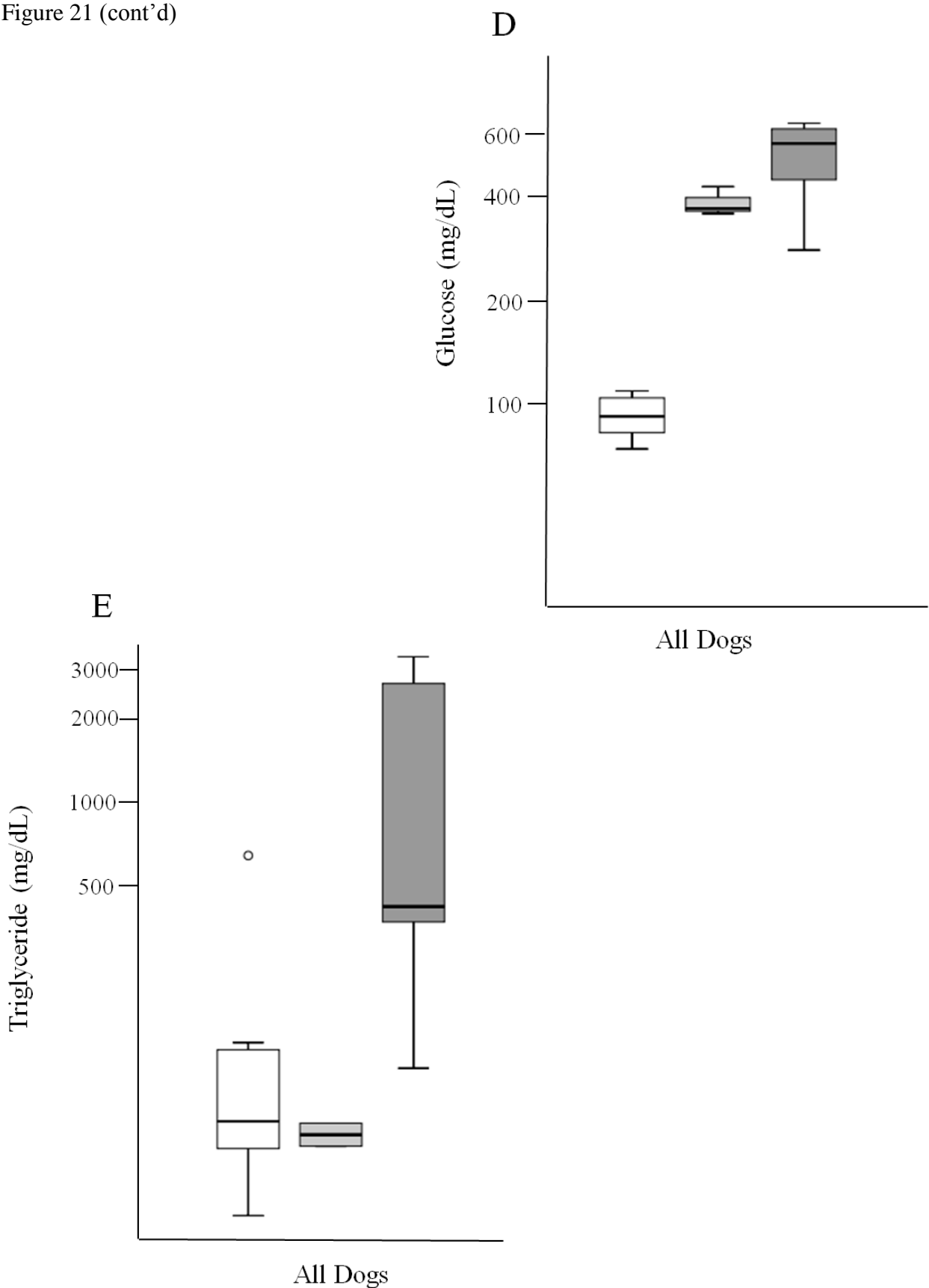
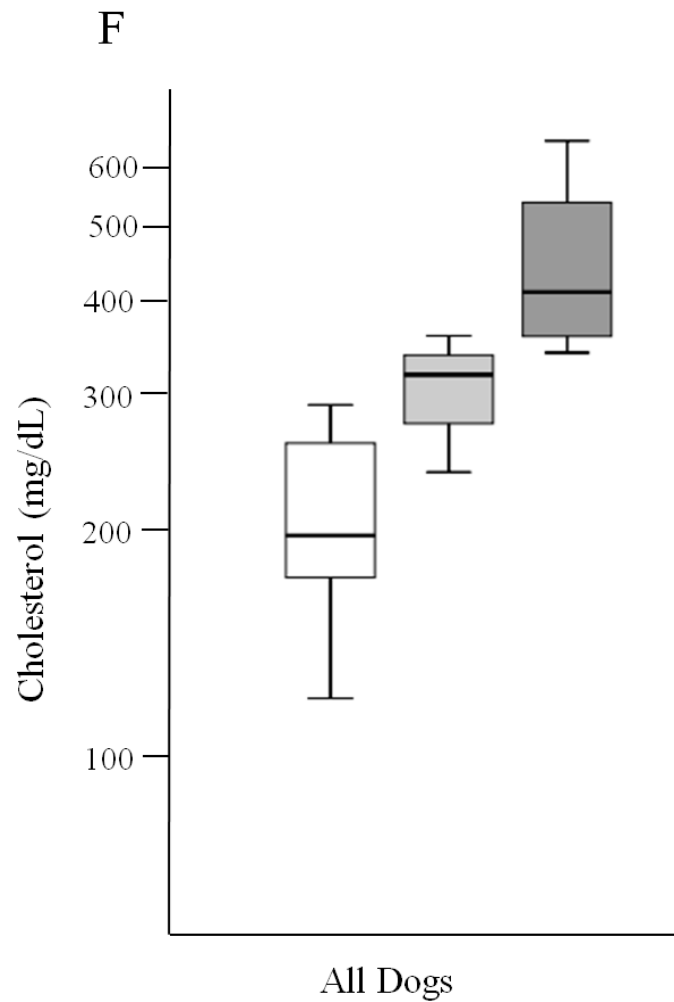


Figure 21 (cont'd)

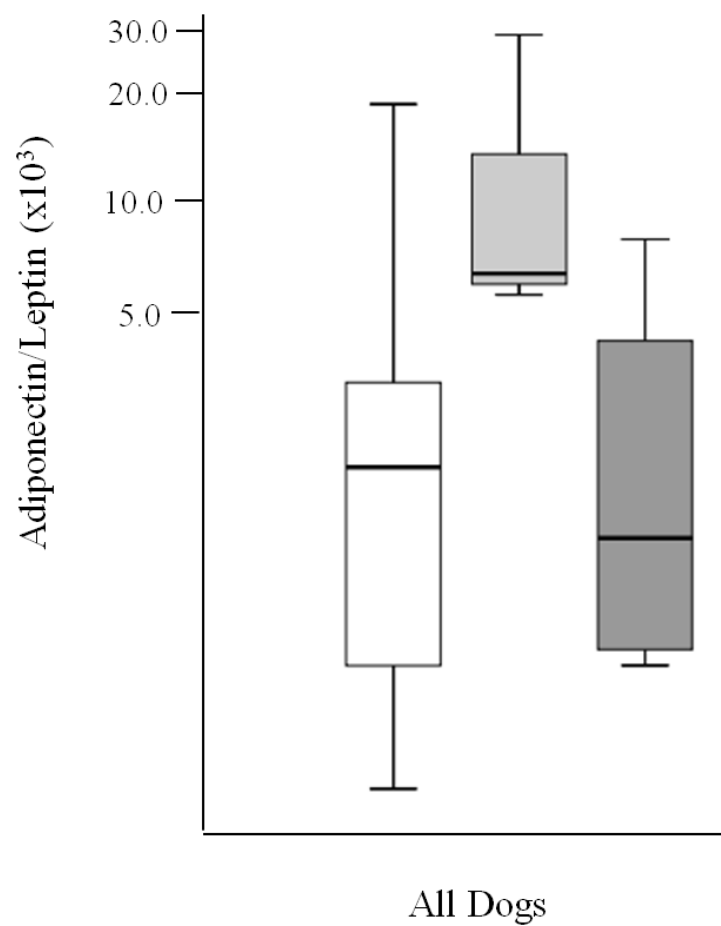


Adiponectin to leptin ratio in dogs with DM

The ratio of serum concentrations of adiponectin to serum concentration of leptin (Mean; 95% CI) was significantly ($P = 0.043$) higher in dogs with UDM (10.07×10^3 ; 2.46×10^3 , 41.18×10^3) compared to healthy dogs (1.41×10^3 ; 0.78×10^3 , 2.55×10^3). The ratio in dogs with DKA (1.57×10^3 ; 0.46×10^3 , 5.31×10^3) was not different from healthy dogs or dogs with UDM (Figure 22).

Figure 22. Adiponectin to leptin ratio in healthy dogs (□), dogs with UDM (◻), and dogs with DKA (■).†

† The ratio is presented on a natural logarithmic scale. The horizontal bar within the box plot indicates the median, the box contains the middle half of the results, the T-bars indicate the range.



Associations among BCS and serum concentrations of hormones, lipids, and glucose in dogs with DM

When all dogs with DM were grouped together, serum concentrations of insulin were significantly correlated to serum concentrations of leptin ($r = 0.93$, $P = 0.003$) and cholesterol ($r = 0.92$, $P = 0.025$). No significant linear correlations between BCS and any of the other variables were revealed.

Associations between hormones, glucose, lipids, and biochemical parameters in dogs with DM

Serum concentrations of triglyceride were significantly correlated to fold increase in concentrations of bilirubin ($r = 0.88$, $P = 0.008$) and activities of ALT ($r = 0.81$, $P = 0.026$) and AST ($r = 0.80$, $P = 0.031$).

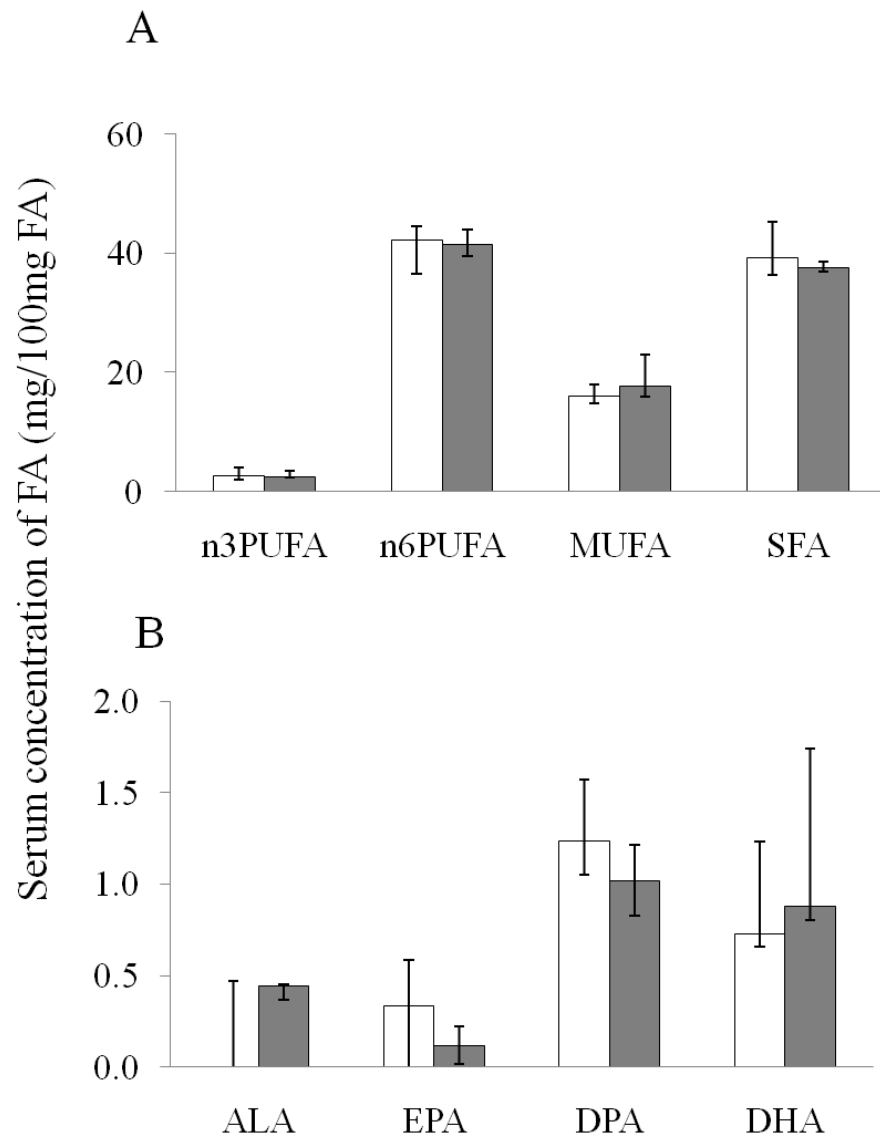
Serum concentrations of FAs in healthy dogs and dogs with DM

Serum FA analysis was available for 8 dogs with DM (4 non-obese and 4 obese) and 11 healthy dogs. No significant differences were revealed in concentrations of any of the individual n3PUFAs or in FA categories between dogs with DM and healthy dogs (Figure 23).

Figure 23. Serum concentration of total FAs within categories (A) and individual n3PUFAs (B) in healthy dogs (□) and dogs with DM (■).†

† Bar represents the median and error bars represent interquartile range.

Figure 23 (cont'd)



Associations between concentrations of total or individual n3PUFAs concentrations of hormones, lipids, and glucose in dogs with DM

An increase of 1 mg/100mg FA in serum concentration of total n3PUFAs was associated with 304% increase in the adjusted geometric mean of serum concentration of leptin, and 342% increase in the adjusted geometric mean of serum concentration of insulin (Table 29). These associations remained significant following inclusion of BCS as an additional covariate in the

models ($P= 0.013$ and $P=0.031$, respectively). No significant associations were revealed between concentrations of the outcome variables and any of the individual n3PUFAs.

Table 29. Associations between total n3PUFAs and adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol in dogs with DM.*

Variable	Mean (95% CI) †	Fold increase (95% CI) ‡	P	Partial η^2
Adiponectin	11.8 $\mu\text{g/mL}$ (2.8, 50.3)	1.53 (0.18, 12.86)	0.6	0.50
Leptin	2.2 ng/mL (0.8, 5.9)	4.04 (1.02, 16.07)	0.048	0.66
Insulin	28 pmol/L (14, 58)	4.42 (1.61, 12.12)	0.013	0.74
Glucose	26.5 mmol/L (21.0, 33.5)	1.16 (0.83, 1.63)	0.3	0.21
Triglyceride	429 mg/dL (35, 5329)	5.26 (0.10, 274.23)	0.3	0.37
Cholesterol	374 mg/dL (280, 499)	1.38 (0.92, 2.07)	0.093	0.55

* Covariate in the GLM is total n3PUFAs.

† Covariate in the model is evaluated at the following value: total n3PUFAs = 2.78 mg/100mg FA.

‡ Fold increases and 95% CI in adjusted geometric means of serum concentration of each variable with an increase of 1 mg/100 mg FA in serum concentration of total n3PUFAs.

Associations between concentrations of total or individual n3PUFA and biochemical parameters in dogs with DM

No significant associations were revealed.

2.3. Discussion

2.3.1. *Feline hepatic lipidosis*

Cats with HL were shown to have increased concentrations of adiponectin and leptin. Concentrations of adiponectin were shown to be increased in cats with liver disease, either HL or other liver disease, while, concentrations of leptin were increased in cats with HL but not in cats with other liver disease. Cats with HL had increased concentrations of glucose with unchanged concentrations insulin, while cats with other liver disease had decreased insulin concentrations with unchanged glucose concentrations. Serum concentrations of triglyceride were increased in cats with HL but not in cats with other liver disease and concentrations of cholesterol were decreased in cats with any liver disease (Table 30).

Adiponectin

This study demonstrated elevated serum concentrations of adiponectin in cats with HL. This finding seems to disagree with the anti-steatotic effect of adiponectin as well as with reports of decreased serum adiponectin concentrations in human patients with NAFLD and rodent models of the disease (Xu et al., 2003; Hui et al., 2004; Targher et al., 2004; Bugianesi et al., 2005; Musso et al., 2005; Pagano et al., 2005; Baranova et al., 2006; Yoneda et al., 2007). However, unlike human NAFLD, which is commonly asymptomatic and only rarely progresses to advanced liver disease (Cave et al., 2007); most cases of feline HL, including the cats in the present study, display evident clinical signs and severe cholestasis at the time of diagnosis (Center, 2005; Armstrong and Blanchard, 2009).

Pathologic changes associated with liver disease in general may have an important effect on adiponectin concentration. This is supported by the finding of increased serum concentration of adiponectin in all cats with liver disease. In addition, in contrast to the inverse relationship between serum adiponectin concentrations and body condition as well the inverse relationships between adiponectin and insulin, leptin, or triglyceride concentrations shown among the healthy cats in this study and in previous reports in other species (Yu and Ginsberg, 2005; Hoenig et al., 2007; Ishioka et al., 2009); none of these associations was present among cats with liver disease. This difference between healthy cats and cats with liver disease suggests that physiological mechanisms that normally determine circulating adiponectin concentrations may be impaired in a state of liver disease. In agreement with the findings of this study, increased serum concentrations of adiponectin have been reported in human patients with chronic liver disease accompanied by cholestasis or cirrhosis (Tietge et al., 2004; Tacke et al., 2005), and in animal models of acute liver injury (Yoda-Murakami et al., 2001; Wolf et al., 2006).

Different mechanisms may lead to increased serum concentrations of adiponectin in cats with liver disease. One potential mechanism is decreased excretion of adiponectin in the bile due to cholestasis. Increased serum adiponectin concentrations have been demonstrated in biliary diseases in humans and shortly following bile duct ligation in mice (Tacke et al., 2005). Another possibility is production of adiponectin in the injured liver, potentially as a protective mechanism. Although adiponectin is normally produced predominantly in the adipose tissue, adiponectin protein was detected in the endothelial cells in the liver following induction of acute hepatic failure in mice (Wolf et al., 2006), and adiponectin mRNA expression was detected in the liver of carbon tetrachloride treated mice (Yoda-Murakami et al., 2001). In addition, decreased hepatic function and altered hemodynamics have been shown to result in decreased

hepatic extraction of adiponectin from the circulation in human patients with cirrhosis (Tietge et al., 2004). In the present study, serum concentrations of adiponectin were directly related to serum ALT activity but not to serum ALP activity among cats with liver disease. These findings suggest that hepatocellular injury is more important than cholestasis in the mechanisms leading to increased circulating adiponectin in these cats.

Additional potential mechanisms that may contribute to the increase in serum concentrations of adiponectin in cats with HL specifically include the presence of hepatic resistance to adiponectin action, as has been suggested in a model of NAFLD in mice (Larter et al., 2008) or a compensatory mechanism to prevent insulin resistance associated with hyperlipidemia, as has been suggested in a recent experimental study that documented increased concentrations of adiponectin following lipid infusion and development of hepatic steatosis in cats (Zini et al., 2010).

Leptin

The increase in serum concentrations of leptin in cats with HL, but not in cats with other liver disease, suggests that this alteration is associated specifically with HL rather than liver disease in general. In addition, serum concentrations of leptin were directly related to serum ALP activity in cats with liver disease. Since ALP activity is an indicator of cholestasis related to lipid accumulation in the liver, this finding may suggest a direct relationship of leptin and hepatic lipid content in cats with liver disease. In agreement with the findings in this study, an early study in humans demonstrated increased serum concentrations of leptin as well as a direct relationship between leptin concentration and liver steatosis in patients with NAFLD (Chitturi et al., 2002).

However, following studies in patients with NAFLD failed to show a significant change in leptin concentrations (Chalasani et al., 2003; Musso et al., 2005).

Serum concentrations of leptin were directly related to body condition in cats with liver disease, similar to findings in healthy cats in this study and in previous reports (Backus et al., 2000; Appleton et al., 2002; Hoenig et al., 2007), suggesting at least partial preservation of the mechanisms that normally determine circulating concentrations of leptin. In view of the anti-steatotic effect of leptin, its increased concentrations in HL may indicate exacerbation of resistance to leptin that occurs in healthy obese cats. Hepatic leptin resistance may lead to progression of hepatic steatosis despite increased leptin concentrations. Additional factors that potentially contribute to increase leptin concentrations in human NAFLD include the presence of hepatic inflammation and chronic hyperinsulinemia (Chitturi et al., 2002). However, neither of these pathologic processes is an important feature of the disease in cats and therefore is unlikely to be involved in the development of hyperleptinemia in feline HL.

Insulin

Serum concentrations of insulin were decreased in all cats with liver disease while concentrations of glucose were increased. These findings are suggestive of insulin deficiency and are consistent with a previous report of decreased insulin concentration in cats with naturally-occurring liver disease (HL or cholangiohepatitis) (Brown et al., 2000a), as well as the finding of deterioration of glucose tolerance in obese cats at the time of development of HL following rapid weight loss (Biourge et al., 1997). It was suggested that normal metabolic adaptation to

prolonged fasting in cats with HL leads to decreased insulin secretion, decreased glucose utilization, and increased hepatic gluconeogenesis.

Unlike the findings in cats in the present study, human NAFLD is associated with increased serum concentrations of insulin due to insulin resistance. This difference may be explained by the fact that most cats are presented following a period of partial or complete anorexia, whereas human NAFLD is usually asymptomatic at the time of diagnosis.

Interestingly, while concentrations of insulin among cats with other liver disease were lower than healthy cats, concentrations among cats with HL were not different from healthy cats. Higher concentrations of insulin in cats with HL compared to cats with other liver disease may be due to presence of obesity-related insulin resistance and elevated insulin concentrations in many of these cats prior to weight loss and development of HL (Biourge et al., 1997; Appleton et al., 2002).

Triglyceride

Serum concentrations of triglyceride in cats with HL were increased compared to healthy cats both in lean and overweight cats, while there were no differences in serum concentrations of cholesterol. These findings are in agreement with previous studies in cats with naturally-occurring and experimentally-induced HL, as well as human NAFLD (Brown et al., 2000a; Blanchard et al., 2004; Bugianesi et al., 2005). Moreover, among the non-obese cats group in the present study, cats with HL alone had higher concentrations of triglycerides compared to cats with HL and concurrent disease as well as cats with other liver disease, indicating association between hypertriglyceridemia and HL particularly, rather than liver disease in general. Increased

triglyceride concentration in HL is the result of a combination of increased assembly and secretion of VLDL from the liver due to increased FAs mobilization from peripheral adipose tissue, as well as decreased VLDL catabolism by lipoprotein lipase, possibly due to insulin deficiency or by hepatic lipase, possibly due to altered environment in the liver. Hepatic LDL clearance may also be impaired although the number of LDL receptors was not found to be decreased (Pazak et al., 1998; Blanchard et al., 2004).

Serum concentrations of triglycerides were inversely related to serum concentration of adiponectin among healthy cats, consistent with the hypolipidemic role of adiponectin and in agreement with findings in humans (Yu and Ginsberg, 2005) as well as the recently reported decreased adiponectin in cats with hypertriglyceridemia (Hatano et al., 2010). In contrast, no association between serum concentrations of triglyceride and adiponectin was present among cats with liver disease, suggesting impairment of the mechanisms by which adiponectin normally exerts its hypolipidemic effect. Similar to healthy cats, serum concentration of triglyceride were directly related to serum concentrations of insulin in cats with liver disease, suggesting preservation of the association of hyperlipidemia and insulin concentrations in a state of liver disease.

Fatty acids

Serum FA profile was altered in cats with HL. Percent of MUFAs and n3PUFAs were higher while percent n6PUFAs was lower in serum of cats with HL than in healthy cats. Significant increases were revealed in the longer chain n3PUFAs (DPA and DHA) but not the in the shorter chain n3PUFAs (ALA and EPA). No difference was revealed in percent SFAs

between cats with HL and healthy cats. An earlier study (Hall et al., 1997) that documented FA composition of adipose tissue and liver from cats with HL reported a lower percent of n6PUFAs and higher percent of MUFAs compared to healthy controls both in adipose tissue and in the liver, similar to the findings in serum in the present study. However, increased percent of SFAs and decreased percent of n3PUFAs was found in adipose tissue and liver in the earlier study, different from the findings in serum in the present study. The reason for the differences in percent n3PUFA and SFAs between the findings in tissues (adipose and liver) and serum is unknown, but due to the prolonged anorexia and catabolic state of cats with HL, the serum FA profile most likely represents FAs mobilization from adipose tissue rather than dietary FAs and it may be speculated that the release of n3PUFAs from adipose tissue at times of accelerated lipolysis is preferred over the release of SFAs. The higher concentrations of DHA and DPA specifically, would suggest that the release of these longer chain n3PUFA is especially favored. This idea is supported by a study (Lin and Connor, 1990) that showed higher adipose tissue incorporation of ALA compared to EPA and DHA when diets with similar concentrations were fed to rabbits. It was suggested that adipose tissue may have limited tolerance or ability for storing these long chain PUFAs.

The negative association of n3PUFAs, and more specifically DPA, with serum concentrations of triglyceride in obese cats suggests a beneficial triglyceride-lowering effect of this FA in obese cats with HL similar to obese healthy cats. However, unlike the findings in healthy cats, a negative association between n3PUFAs and adiponectin was found in cats with HL, therefore, this lowering-effect of n3PUFAs in HL does not appear to be mediated through adiponectin.

The findings of negative associations between n3PUFAs and liver enzymes in cats with HL may suggest a protecting effect of n3PUFAs on the liver, possibly through attenuation of lipidosis. The negative association between n3PUFAs and adiponectin in cats with HL may be mediated through their associations with liver enzymes, since a positive association between liver enzymes and adiponectin was also present.

Table 30. A summary of associations between serum concentrations of adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol in cats with liver disease.

	HL	HL with concurrent disease	Other liver disease
Adiponectin	↑	↑	↑
Leptin	↑	↑	N
Insulin	N	N	↓
Glucose	↑	↑	N
Triglyceride	↑	↑/N	N
Cholesterol	↓?	↓?	↓?

↑: increase, ↓: decrease, or N: no difference in serum concentrations compared to healthy cats

2.3.2. Canine hypothyroidism

Hypothyroid dogs were shown to have increased serum concentrations of adiponectin and leptin. Serum concentrations of insulin were increased while concentrations of glucose were unchanged. Concentrations of triglyceride and cholesterol were increased (Table 31).

Leptin

This study shows higher serum leptin concentrations in hypothyroid compared to healthy dogs. Human and animal studies yielded conflicting results regarding leptin concentrations in hypothyroidism. The findings in our study are in agreement with a study (Pinkney et al., 1998) demonstrating hyperleptinemia in hypothyroid human patients, most of which (20 of 22) were diagnosed with autoimmune thyroiditis, which is also the most common etiology of canine hypothyroidism.

Several factors may contribute to the occurrence of hyperleptinemia in hypothyroidism. Thyroid hormone deficiency may directly affect leptin concentration. This potential mechanism is supported by the significant negative correlation between leptin and total T4 found in our hypothyroid group and by previous findings in other species. Hypothyroid rats have increased leptin serum concentration and expression in adipocytes (Fain et al., 1997). In humans and rats, leptin has been shown to up-regulate Thyrotropin-Releasing Hormone expression, and in the rat it also directly stimulates growth and secretion from the thyroid gland (Zimmermann-Belsing et al., 2003). In addition, T3 enhances the loss of leptin mRNA in rat adipocytes (Fain and Bahouth, 1998). Therefore, it is thought that leptin has a role in stimulating the hypothalamic-

pituitary-thyroid axis in response to low thyroid hormone concentrations under physiologic conditions. In hypothyroidism this mechanism is amplified, and accordingly, leptin concentration is increased. Reduced leptin turnover and degradation may also contribute to hyperleptinemia associated with hypothyroidism (Houseknecht et al., 1996).

Obesity and hyperinsulinemia are possible additional contributors to the hyperleptinemia found in the hypothyroid dogs. Obesity is reported to be associated with hyperleptinemia in dogs (Ishioka et al., 2002b; Sagawa et al., 2002; Jeusette et al., 2005) and was probably an important but not the sole factor causing hyperleptinemia in our group of hypothyroid dogs. Although leptin concentration was positively correlated to BCS in the hypothyroid group, the difference in leptin between the groups remained significant after adjusting for BCS. The finding of a nearly significant interaction between hypothyroidism status and BCS suggests that the effect of hypothyroidism on serum leptin concentration varies in dogs of different body condition. Nevertheless, leptin concentrations were higher in the hypothyroid dogs compared to the normal dogs in all BCS levels, although the magnitude of difference between the groups was smaller in the higher compared to the lower BCS levels. Hyperinsulinemia may also cause hyperleptinemia since insulin increases the expression of leptin mRNA in adipocytes (Cusin et al., 1995). Our study, however, cannot support this mechanism, as no correlation was found between insulin and leptin in the hypothyroid dogs and the difference in leptin between the groups remained significant after adjusting for insulin. Hyperlipidemia was not an important factor causing hyperleptinemia in our study, as no correlation was found between leptin and cholesterol or triglyceride concentrations in the hypothyroid dogs and the difference in leptin between the groups remained significant after adjusting for triglyceride. This is in agreement with findings in

humans suggesting that hyperleptinemia in patients with hyperlipidemia reflects body fat content and is not related to serum lipid concentrations (Haluzik et al., 2000).

Insulin and glucose

Our study demonstrated hyperinsulinemia coupled with normoglycemia in hypothyroid dogs. This suggests that canine hypothyroidism is associated with insulin resistance, in which a compensatory increase in insulin concentration is sufficient to maintain normoglycemia. The occurrence of insulin resistance in canine hypothyroidism was previously demonstrated by prolonged insulin response to glucose in experimentally radioiodine-induced canine hypothyroidism (Renauld et al., 1982) and was suggested to occur in three dogs with naturally-occurring hypothyroidism and concurrent DM that required high doses of insulin prior to T4 replacement therapy (Ford et al., 1993). Studies on glucose metabolism in human hypothyroidism are scarce and report inconsistent results. Our findings in dogs are consistent with the observed decrease in glucose utilization in hypothyroid compared to euthyroid rats (Okajima and Ui, 1979).

Several factors may cause insulin resistance and hyperinsulinemia in hypothyroidism. Thyroid hormone deficiency may directly lead to insulin resistance. The GLUT4 gene contains a T3-responsive element and accordingly GLUT4 expression is decreased in rats with thyroid hormone deficiency (Torrance et al., 1997). In addition, thyroid hormones modulate the activity of some regulatory enzymes in glucose metabolism (Rochon et al., 2003). Such disruptions in glucose homeostasis may induce compensatory hyperinsulinemia to maintain normoglycemia. Another potential direct effect of hypothyroidism is decreased insulin clearance resulting in

hyperinsulinemia, as has been shown in hypothyroid human patients (Dimitriadis et al., 2006). Our study, however, cannot support a direct effect of thyroid hormone deficiency on insulin sensitivity or clearance as no correlation was found between total T4 and insulin concentrations in the hypothyroid dogs.

Obesity and hyperlipidemia are two main features of canine hypothyroidism, both of which have been associated with hyperinsulinemia in humans as well as dogs (Kahn and Flier, 2000; Lewis et al., 2002; Jeusette et al., 2005). Obesity may lead to insulin resistance through several mechanisms including: down regulation of GLUT4 caused by accumulation of fat in the adipocyte, interference with insulin signalling in muscle and impairment of insulin-mediated suppression of glucose production in the liver, and resistance to leptin. Hyperlipidemia may lead to impairment of insulin receptor binding affinity, down-regulation of the receptor, and a post-receptor defect in insulin action (Kahn and Flier, 2000; Lewis et al., 2002). Hyperlipidemia may have played an important role in the occurrence of hyperinsulinemia in our hypothyroid dogs as serum triglyceride and cholesterol concentrations were strongly positively correlated to insulin concentrations. Nevertheless, neither hyperlipidemia nor obesity and its associated leptin resistance can be exclusively accounted for the hyperinsulinemia in the hypothyroid dog group as the difference in insulin between the two study groups remained significant after adjusting for BCS, triglyceride, and leptin.

Adiponectin

This study revealed increased serum concentrations of adiponectin in dogs with naturally-occurring hypothyroidism. This was a surprising finding in view of direct relationship between

serum concentrations of adiponectin and thyroid hormones reported in euthyroid healthy human subjects as well hypothyroid and hyperthyroid human patients (Fernandez-Real et al., 2003; Yaturu et al., 2004; Yu et al., 2006; Hsieh and Wang, 2008; Lin et al., 2010), suggesting a stimulatory effect of thyroid hormones on adiponectin production. Nevertheless, most previous reports in human patients with thyroid dysfunction as well as rodent models report no difference in serum concentration of adiponectin compared to euthyroid subjects (Iglesias et al., 2003; Santini et al., 2004; Nagasaki et al., 2005; Altinova et al., 2006; Yu et al., 2006; Aragao et al., 2007; Bossowski et al., 2010; Sieminska et al., 2010), while some (Dimitriadis et al., 2006; Pontikides and Krassas, 2007) revealed decreased adiponectin in hypothyroidism.

Similar to the findings in the present study, increased serum concentrations of adiponectin were found in experimentally-induced hypothyroid rats (Kokkinos et al., 2007). However, the increase in adiponectin in that study was considered secondary to weight loss that accompanied the induction of hypothyroidism in this model and therefore, most likely does not accurately represent the metabolic changes in the generally obese hypothyroid dogs in the present study. An *in vitro* study using rat subcutaneous adipose tissue explants demonstrated an inhibitory effect of T3 on adiponectin mRNA expression (Cabanelas et al., 2010). The inverse relationship between concentrations of adiponectin and T4 demonstrated among the hypothyroid dogs in our study could potentially be consistent with such an inhibitory effect, as the decrease in thyroid hormones in hypothyroidism may lead to attenuation of an inhibitory effect on adiponectin synthesis and elevation of serum adiponectin concentrations. However, stimulation of adiponectin production and augmentation of its metabolic effects would be expected to result in improvement of insulin sensitivity and attenuation of hyperlipidemia, contrary to the findings

of hyperlipidemia and elevated serum concentrations of insulin in hypothyroid dogs in the present as well as previous reports (Panciera, 1994; Dixon et al., 1999).

The occurrence of elevated serum concentrations of adiponectin together with metabolic alterations that are consistent with dysfunction of adiponectin in the hypothyroid dogs are suggestive of the presence of resistance to adiponectin. The presence of an inverse relationship between adiponectin and triglyceride among the healthy dogs but not among the hypothyroid dogs further support the notion of uncoupling of this hormone signals in hypothyroidism. The presence of resistance to adiponectin has been recently suggested in association with insulin resistance and hyperlipidemia in humans and rodent models (Mullen et al., 2009; Li et al., 2010; Yi et al., 2011), but it is unclear whether the resistance to adiponectin is a cause or a result of these metabolic alterations. Mechanisms leading to the development of adiponectin resistance are still not completely understood, but impairment of adiponectin receptors function and/or post-receptor transcriptional events has been described. Accordingly, increased concentrations of adiponectin with no response to exogenous adiponectin administrations have been described in a model of insulin-resistance (Lin et al., 2007), reduction of mRNA expression of adiponectin receptors in the liver and skeletal muscle has been demonstrated in insulin-resistant rodents (Tsuchida et al., 2004), and a direct inhibitory effect of insulin on expression of ADR1 in vitro was found in mouse skeletal muscle cells (Inukai et al., 2005). In contrast, in humans a positive association between serum insulin concentration and ADR1 has been found in vivo, while no effect of insulin on the receptor expression in myotubes was found in vitro (Staiger et al., 2004a). In another rat model of insulin resistance, increased serum concentrations of adiponectin were associated with overexpression of adiponectin receptors in the skeletal muscle, but impaired downstream signaling (Rodriguez et al., 2008). These conflicting results may be partly explained

by differences in adiponectin regulation between species or differences in study design and models.

Triglyceride and cholesterol

Similar to previous reports (Panciera, 1994; Dixon et al., 1999), serum cholesterol and triglyceride concentrations were significantly higher in the hypothyroid compared to the normal dogs in our study. However, as dogs with elevated serum concentrations of triglycerides and/or cholesterol may be more likely to be screened for hypothyroidism, these differences may be at least partly due to a selection bias. Nevertheless, as discussed above, neither hyperinsulinemia, hyperleptinemia, nor hyperadiponectinemia in the hypothyroid dog group was the result of hyperlipidemia alone. Dogs with hypothyroidism have been shown to have increased VLDL, LDL, and HDL (Rogers et al., 1975; Barrie et al., 1993). Thyroid hormones stimulate all aspects of lipid metabolism, including synthesis, mobilization, and degradation. Both the synthesis and degradation of lipids are depressed in hypothyroidism, with degradation affected more than synthesis. Thyroid hormone deficiency-induced decrease in hepatic LDL receptor activity and reduced activities of lipoprotein lipase and hepatic lipase are proposed as underlying mechanisms responsible for the lipoprotein cholesterol abnormalities identified in hypothyroid dogs (Feldman and Nelson, 2004c).

Fatty acids

Serum concentrations of total n3PUFAs as well as concentrations of each of the individual n3PUFAs were increased in hypothyroid dogs. An experimental study in thyroidectomized dogs (Campbell and Davis, 1990) demonstrated increased serum concentrations of EPA, but decreased concentrations of DHA in the hypothyroid dogs. Similarly, concentrations of EPA and DPA were increased in liver mitochondria of hypothyroid rats compared to controls, and concentrations of DHA were decreased and concentrations of ALA were unchanged (Raederstorff et al., 1991). In these two studies, concentrations of AA were decreased in hypothyroid animals, while concentrations of LA were increased in hypothyroid rats. The n3- and n6PUFAs are metabolized by the same enzyme sequence and a competitive inhibition occurs between the two series at each step in the normal metabolism of these PUFAs. Hypothyroidism suppresses the activities of $\Delta 5$ and $\Delta 6$ desaturases as well as elongase. Therefore, it was suggested that the competition between n3 and n6PUFAs favored the synthesis of n3PUFAs up to DPA in rats and EPA in dogs (Raederstorff et al., 1991). In the present study, metabolism of n3PUFAs seems to be favored up to DHA. The reason for the opposite changes in DHA concentrations in hypothyroid dogs between the present and previous studies is unclear, but may be the different etiology (thyroidectomy vs. naturally-occurring hypothyroidism) and duration of hypothyroidism.

If increases in liver enzymes are indicative of the severity of the hypothyroid state, then the positive associations revealed between the long chain n3PUFAs (EPA and DHA) and liver enzymes may be consistent with more pronounced alterations in n3PUFAs in dogs with a more severe disease.

The present study failed to reveal any associations between n3PUFAs and adipokines in hypothyroid dogs. The sample size of this study may have been a restricting factor.

Table 31. A summary of associations between serum concentrations of adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol in dogs with hypothyroidism.

	Hypothyroidism
Adiponectin	↑
Leptin	↑
Insulin	↑
Glucose	N
Triglyceride	↑
Cholesterol	↑

↑: increase, ↓: decrease, or N: no difference in serum concentrations compared to healthy dogs

2.3.4. Canine and feline diabetes mellitus

Concentrations of leptin were increased in cats and decreased in dogs with UDM. Cats with DKA had increased leptin concentrations while concentrations were unchanged in dogs with DKA. Coupled with hyperglycemia, concentrations of insulin were increased in cats with UDM, but unchanged in cats with DKA, whereas dogs with UDM or DKA had decreased insulin concentrations. Concentrations of triglyceride and cholesterol were increased in cats and dogs with DKA, but only in cats with UDM. Concentrations of adiponectin were unchanged in UDM or DKA both in dogs and cats (Table 32).

Insulin and glucose

The finding of opposite alterations in serum concentrations of insulin in canine and feline DM is a consequence of the different pathogenesis of DM in these two species. The decreased insulin concentration in dogs with UDM is consistent with deficiency of insulin secretion in Type I DM, while the increased insulin concentration in cats with UDM is indicative of insulin resistance in Type II DM. Cats with DKA did not have increased insulin concentrations, most likely due to β -cell failure as a result of glucose toxicity in advanced stage of type II DM.

Leptin

The opposite alterations in serum concentrations of leptin between dogs and cats with UDM are most likely due to the different pathogenesis of DM resulting in different concentrations of insulin.

The finding of decreased leptin concentrations in dogs with UDM is in agreement with a recent report in dogs (Nishii et al., 2010) and previous reports in humans (Kirel et al., 2000; Soliman et al., 2002) or rat models (Havel et al., 1998; Sivitz et al., 1998) of type I DM. The finding of increased leptin concentrations in cats with UDM in the present study is in agreement with one study reporting increased concentrations of leptin in human type II DM (Widjaja et al., 1997). However, in contrast to the consistent findings on leptin concentrations in type I DM, other studies on type II DM report controversial results; some demonstrated decreased concentrations (Roden et al., 2000; Sivitz et al., 2003; Al-Shoumer et al., 2008), while others failed to reveal any difference in leptin concentrations (Verrotti et al., 1998; Rojo-Martinez et al., 2000).

The decrease in leptin in dogs with DM is likely to be the result of insulin deficiency, as insulin is known to act as a leptin secretagogue in humans (Lee and Fried, 2009) and administration of insulin to healthy dogs was also shown to increase circulating concentrations of leptin (Ishioka et al., 2005). This potential mechanism for the hypoleptinemia in diabetic dogs is supported by the strong positive association between serum concentrations of insulin and leptin revealed in the present study. The same mechanism may explain the increased serum leptin concentrations in cats with UDM as hyperleptinemia was accompanied by hyperinsulinemia in these cats. Although the increase in insulin concentrations is generally considered to result from

resistance to insulin in its target tissue, it is possible that the adipocytes are able to respond to the increased concentrations of insulin by enhanced production of leptin. The controversial findings on leptin concentrations in humans with type II DM may be explained, at least partially, by the changes in capacity of β cells to secrete insulin as the disease progresses. Earlier stages of the disease are characterized predominantly by insulin resistance, while β cell failure and decreased insulin secretion occur at later stages. Indeed, hyperleptinemia in human patients with type II DM was accompanied by hyperinsulinemia in one study (Widjaja et al., 1997), while decreased leptin concentrations were reported in human patients with decreased insulin secretion (Roden et al., 2000; Sivitz et al., 2003; Al-Shoumer et al., 2008).

Concentrations of leptin were higher in cats with DKA than healthy cats or cats with UDM. In dogs with DKA, concentrations of leptin were not decreased compared to healthy dogs, in contrast to the findings in dogs with UDM. These findings are in agreement with a study that demonstrated elevated leptin concentration in human patients with DKA prior to treatment (Nakamura et al., 1999).

In cats with DKA, serum concentrations of leptin were higher compared to healthy cats, although there was no difference in concentrations of insulin. In dogs with DKA, concentrations of leptin were unchanged compared to healthy dogs, but insulin concentrations were decreased. In both cases, concentrations of leptin are inappropriately high relative to concentrations of insulin. Several potential explanations for this elevation in serum concentrations of leptin in DKA may be offered. Severe hyperglycemia and ketonemia lead to dehydration, resulting in prerenal hypovolemia and a decrease in glomerular filtration rate. Serum leptin concentrations were demonstrated to be increased in human patients with renal insufficiency (Merabet et al., 1997); therefore potential reduction in glomerular filtration rate in dogs and cats with DKA may

lead to the elevation of serum leptin concentrations. This potential mechanism is supported by the finding of positive associations of serum concentrations of creatinine and BUN to concentrations of leptin in cats with DKA. In addition, induction of leptin production and release by glucose may be stimulated due to the marked hyperglycemia in DKA. Concentrations of glucose were higher in dogs and cats with DKA than UDM, although these differences did not reach statistical significance. And lastly, marked elevation of serum cortisol concentration was demonstrated in humans patients with DKA (Nakamura et al., 1999) and glucocorticoid are known to increase synthesis and secretion of leptin.

Triglyceride and cholesterol

The elevation in serum concentrations of triglyceride and cholesterol both in dogs and in cats with DM are consistent with previous reports (Feldman and Nelson, 2004a; Hume et al., 2006).

Hypercholesterolemia consists of increased LDL-cholesterol due increased LDL synthesis and reduced LDL receptors activity. HDL-cholesterol is often low in diabetic humans due to accelerated catabolism. Dogs with DM have been shown to have increased VLDL and HDL (Barrie et al., 1993). Hypertriglyceridemia results from impaired lipoprotein lipase activity due to insulin deficiency, resulting in decreased metabolism of VLDL and chylomicrons. In addition, increased circulating concentrations of free FAs lead to increased hepatic VLDL production. The elevation of serum triglyceride concentrations in cats but not in dogs with UDM may be related to the fact that some cats with UDM have type II DM are obese at presentation, whereas practically all dogs with UDM have type I DM, most of those dogs are lean to

underweight at presentation. Moreover, DKA is associated with concurrent disease in most cases (Hume et al., 2006); some of these diseases, such as pancreatitis and hyperadrenocorticism, may induce hyperlipidemia as well.

The finding of positive associations between serum concentrations of triglyceride and liver enzymes in dogs with DM is consistent with progression of hepatic lipid accrual due to insulin deficiency-induced lipolysis and increased mobilization of FAs to the liver.

Adiponectin

No significant alterations in serum concentrations of adiponectin in dogs or cats with UDM or DKA were revealed in the present studies. In humans, type I diabetes is associated with increased serum concentrations of adiponectin (Frystyk et al., 2005; Celi et al., 2006; Barnes et al., 2008; Leth et al., 2008; Abi Khalil et al., 2011), whereas type II DM is associated with decreased adiponectin concentrations (Hotta et al., 2000; Sheng and Yang, 2008).

The decrease in concentrations of adiponectin in human patients with type II DM is related to obesity that is characteristic of this type of DM. Obesity and its associated hypoadiponectinemia are considered to play an important role in the development of insulin resistance and its progression to type II diabetes. The elevation of adiponectin concentration in human patients with type I DM is unclear. Suggested mechanisms include decreased renal clearance of adiponectin due to nephropathy (Saraheimo et al., 2005) or induced adiponectin production by insulin treatment (Blumer et al., 2008). However, increased adiponectin concentrations have also been documented in patients with type I DM that was not complicated by nephropathy as well as in patients prior to therapy (Abi Khalil et al., 2011). Therefore,

additional mechanisms are likely involved in this alteration in adiponectin concentrations in human type I DM.

Interestingly, mean adiponectin concentration was higher in dogs and lower in cats with UDM compared to healthy controls, but these differences were not statistically significant. It is possible that a larger sample size is required to reveal potential differences in serum concentrations of adiponectin compared to leptin as the variance in adiponectin concentrations was higher than the variance in leptin.

The negative association between serum concentrations of adiponectin and triglyceride in cats with DM suggests that the beneficial hypolipidemic effect of adiponectin is preserved in cats with overt DM.

Fatty Acids

Diabetic cats had increased serum concentrations of total n3PUFAs as well as increased concentrations of ALA and DHA. These results are in agreement with a study in children that reported increases in ALA and DHA in obese children with insulin resistance compared to obese or lean children with normal insulin sensitivity (Gil-Campos et al., 2008). Dietary intake of FAs was not considered the reason for this difference in that study. It is possible that the serum FA profile in cats with DM more closely represents FA mobilization from adipose tissue rather than dietary FAs, similar to cats with HL. Although prolonged anorexia is a less consistent presenting feature of feline DM, FAs mobilization from the adipose tissue is accelerated due to decreased insulin action, as a result of insulin resistance or insulin deficiency. No significant alterations

were revealed in serum FA profile of diabetic dogs. These results did not provide support for insulin deficiency as an important determinant of FA metabolism in dogs.

Total n3PUFAs concentrations were positively associated with concentrations of insulin and leptin in diabetic dogs. These results suggest a positive effect of n3PUFAs on insulin and leptin secretion. Cellular increase of n3PUFAs through transgenic manipulation of isolated pancreatic islets has been recently demonstrated to stimulate insulin secretion (Wei et al., 2010). A potential mechanism suggested was reduction in prostaglandin E2 synthesis in the transgenic cells due to decreased availability of AA; prostaglandin E2 has been demonstrated as a negative regulator of insulin secretion. In addition, this study showed that the transgenic islet cells were resistant to cytokine induced cell death. Moreover, a clinical study in humans (Norris et al., 2007) established that daily consumption of n3PUFAs in early life can have benefits against the development of type I diabetes and islet autoimmunity. It may be speculated that higher concentrations of n3PUFAs in diabetic dogs in the present study had a protective effect on islet cells as well as a beneficial effect on insulin secretion. A positive effect of n3PUFAs on leptin production may be mediated through the effect of insulin on leptin secretion or may occur directly through the effect of n3PUFAs on PPAR γ to increase leptin secretion, similar to the suggested mechanism in healthy dogs.

The negative associations between n3PUFAs and liver enzymes and bilirubin in cats with DM, similar to the findings in cats with HL, suggests a protective effect of these FAs on the liver, potentially through decreased hepatic fat accrual that accompanies DM.

Table 32. A summary of associations between serum concentrations of adiponectin, leptin, insulin, glucose, triglyceride, and cholesterol in cats and dogs with DM.

	UDM		DKA	
	Cats	Dogs	Cats	Dogs
Adiponectin	N	N	N	N
Leptin	↑	↓	↑	N
Insulin	↑	↓	N	↓
Glucose	↑	↑	↑	↑
Triglyceride	↑	N	↑	↑
Cholesterol	↑	N	↑	↑

↑: increase, ↓: decrease, or N: no difference in serum concentrations compared to healthy dog or cats

2.3.5. Summary

Alterations in adipokines concentrations revealed in dogs and cats with naturally-occurring obesity-related diseases were different from the changes expected to be present as a result of obesity and its associated metabolic derangements alone, i.e. insulin resistance, hepatic statosis. These findings suggest that pathologic changes present in dogs and cats with overt diseases associated with obesity may have important effects on concentrations of adipokines. Potential factors include renal clearance and hepatic metabolism of adipokines as well as effect of other hormones, such as insulin, T4, and cortisol, on production and secretion of adipokines.

Serum concentrations of adiponectin were increased in cats with different types of liver disease, while serum concentrations of leptin were increased in cats with HL (either alone or with concurrent disease) but not in cats with other type of liver disease. Therefore, the ratio of adiponectin to leptin was significantly lower in cats with HL than other liver disease and a ratio higher than 1×10^3 indicates that liver disease other than HL is likely to be present.

Canine hypothyroidism was demonstrated to be associated with increased serum leptin and insulin concentrations, suggesting insulin resistance. Although obesity, which is a common feature of hypothyroidism, is known to produce these metabolic alterations, our study suggests that additional factors are likely involved. These include hyperlipidemia leading to hyperinsulinemia and a direct effect of thyroid hormone deficiency promoting hyperleptinemia. In addition, the increased serum concentrations of adiponectin in dogs with hypothyroidism are suggestive of the presence of resistance to adiponectin that could have contributed to the development of hyperlipidemia and insulin resistance in the hypothyroid dogs or alternatively, could be a consequence of these metabolic alterations.

Feline DM was demonstrated to be associated with hyperleptinemia and hyperinsulinemia, whereas canine DM was associated with hypoleptinemia and hypoinsulinemia. These findings are most likely the consequence of the different common etiologies of DM in cats (some type II) and dogs (virtually all type I).

Concentrations of n3PUFAs were generally increased in dogs and cats with overt obesity-related diseases (feline HL, feline DM, and canine hypothyroidism), potentially indicating either increased mobilization of FAs from the adipose tissue or favored n3PUFAs synthesis. Canine DM was not associated with alterations in n3PUFAs concentrations.

In general, the positive effects of n3PUFAs on concentrations of adiponectin and leptin demonstrated in healthy dogs and cats were not preserved in animals with overt diseases. These findings suggest that additional factors related to the specific disease may have stronger effects on adipokines. Nevertheless, n3PUFAs may have had hypolipidemic and anti-steatotic effects that may not be mediated through adipokines.

CHAPTER 3

PRESENT LIMITATIONS AND FUTURE DIRECTIONS

Potential effects of n3PUFAs on secretion of adiponectin and leptin in dogs and cats were suggested by the findings of positive associations between an n3PUFA and serum concentrations of adiponectin and leptin. A cause and effect relationship was established for secretion of adiponectin in non-obese dogs by the demonstration of increased serum concentrations in response to fish oil supplementation. However, such a relationship could not be established for leptin in this group of dogs. In addition, the effect of fish oil supplementation on concentrations of adiponectin and leptin in cats were not evaluated in these studies; therefore, a cause and effect relationship in cats can only be suggested, but was not verified. A study to determine the effect of dietary fish oil supplementation on concentrations of adipokines in cats is warranted. Moreover, a direct effect of EPA to increase secretion of adiponectin from mature canine adipocytes was demonstrated and additional effects that are proposed to contribute to increased adiponectin secretion were shown as well, including an inhibitory effect of EPA on secretion of IL6 from SVCs, an inhibitory effect of PA on secretion of adiponectin from mature adipocytes, and a stimulatory effect of PA on secretion of IL6 from SVCs. In cats, a direct effect of n3PUFAs on secretion of adiponectin from adipose tissue could only be suggested by the findings on a stimulatory effect of AA on secretion of IL6 from SVCs, but a direct effect on secretion of adiponectin from mature adipocytes was not revealed. Determining the direct effect on feline adipocytes during a longer duration of treatment is needed to detect potential delayed effects.

All the studies included client-owned dogs and cats rather than purpose-bred animals. Investigation of a natural population and naturally-occurring disease has a distinct advantage of studying the specific species as well as the specific disease in question, avoiding potential species differences and dissimilarities between a naturally-occurring disease and its model. This

is exemplified by the different findings between dogs and cats in the present studies, including the differential associations between n3PUFAs and adipokines in non-obese and obese cats compared to the similar associations in dogs with variable body condition, the finding of a direct effect of EPA on secretion of adiponectin from mature adipocytes and IL6 from SVCs in canine adipose tissue culture, but lack of direct effects of EPA in feline adipose tissue culture, the stimulatory effect on secretion of IL6 from SVCs by PA in canine culture compared to AA in feline culture, as well as the numerous differences among previous studies in humans and rodent models discussed in the previous chapters.

However, clinical studies entail inherent limitations. The natural population consists of animals of different genders, as well as wide ranges of age, body condition, and stage of disease. Therefore, using such a population results in increasing the degree of variability of the studied outcome (i.e. concentrations of adipokines) that is not directly related to the main subject in question (i.e. the effect of n3PUFA or the effect of the disease). In order to determine the associations between a specific variable of interest and the studied outcome variable, statistical analytic methods that allow for controlling of the effects of other potential variables associated with the outcome variable were used (i.e. General linear model or Generalized estimating equations). Inclusion of multiple covariates in these models increases the sample size required in order to allow detection of significant associations. Therefore, failure to reveal associations in these studies does not necessarily indicate lack of effect of a specific variable (e.g. age, gender, body condition, n3PUFA, a disease), but could also result from insufficient sample size. A-priori calculation of the minimum sample size or post-hoc calculation of the power of a study to discover a statistically significant difference requires knowledge of the clinically significant difference in the outcome variable. Since adipokines have not yet been extensively studied in

clinical studies, such a clinical significance of a difference is unknown at this time. Therefore, sample size may have been insufficient to reveal potential associations or differences between groups in a few of the studies, including differences in concentrations of adiponectin in dogs with DM, and associations between n3PUFA and adipokines in canine hypothyroidism. Repeating these studies with larger groups of dogs and cats is warranted. On the other hand, finding of statistically significant associations or differences in studies with relatively small sample size strongly supports clinical significance of these findings. Moreover, using a diverse population for these studies and inclusion of additional potential modulators of adipokines in these models, allowed determination of the associations of additional variables (i.e body condition, age, and gender) with adipokines as well.

The diseases studied were associated with alterations in concentrations of adipokines that were not related to changes in body condition alone. These changes may be attributed either to the primary pathology of the disease or to secondary pathologic changes that develop with the disease, and therefore could potentially play a role in the pathogenesis of the disease and its complications on one hand, and may serve as indicators for disease development and progression on the other hand.

Cats with HL showed elevations of both adiponectin and leptin, whereas cats with liver disease other than HL had increased adiponectin, but unchanged leptin. These alterations resulted in an adiponectin to leptin ratio that is unchanged in cats with HL (with or without concurrent disease), but is significantly higher in cats with other liver disease compared to healthy cats. Adiponectin to leptin ratio was higher than 1,000 in most cats with liver disease other than HL, while it was lower than this cutoff in most cats that had a component of HL. These findings suggest that when a cat with liver disease is presented, the adiponectin to leptin ratio may serve

as a marker to differentiate between cats that suffer from HL from cats other liver disease. Such a biochemical marker may have an important advantage over the more invasive methods routinely used to determine the presence of HL (i.e. Fine needle aspiration or biopsy of the liver), especially in cases with suspected coagulopathy or when the use of chemical sedation/ tranquilization is undesirable due to the general condition of the cat. Future studies should determine the specificity and sensitivity of the measure of adiponectin to leptin ratio for differentiation between HL and other liver disease in cats with overt liver disease.

In addition, cats with HL had elevated leptin concentrations compared to healthy cats of comparable body condition. This elevation was suggested to be related to the pathology of HL specifically rather than liver disease in general. Therefore, elevation of leptin in an overweight-obese cat that exceeds the value expected by the cat's body condition may be an indicator for the presence of HL. Future studies should determine the specificity and sensitivity of a measure of leptin related to body condition (e.g. leptin to calculated %BF ratio) for detection early HL in obese cats without evident liver disease,

Dogs with UDM showed decreased leptin concentrations while concentrations of leptin in dogs with DKA as well as concentrations of adiponectin both in UDM and with DKA were unchanged. These alterations resulted in an adiponectin to leptin ratio higher than 5,000 in all dogs with UDM, while most dogs with DKA had a ratio lower than 5,000. These findings suggest that the adiponectin to leptin ratio may be a biochemical marker to differentiate DKA and UDM in dogs. The clinical utility of such a marker at this time is questionable as a simple serum or urine test for ketones is readily available. Nevertheless, further investigation is warranted to determine whether the adiponectin to leptin ratio may be more sensitive for detection of imminent DKA prior to increases of serum betahydroxybutiric acid or urinary

acetoacetate, in which case the adiponectin to leptin ratio would be valuable to detect diabetic dogs at risk to develop DKA.

Decreased leptin was revealed in canine DM and elevation of leptin was found in feline DM, and no change in adiponectin was found in either canine or feline DM. Elevations of both adipokines were revealed in feline HL and canine hypothyroidism. The elevations in concentrations of these adipokines may suggest resistance to their actions, decreased clearance, or induced production by tissues other than the adipose tissue. The decrease in leptin in canine DM is more likely a result of the primary pathology (i.e. insulin deficiency), rather than a cause of it, but further investigation is required in order to establish a cause and effect relationship. Overall, these studies did not reveal deficiencies in adipokines as potential causes for the metabolic alterations associated with overt obesity-related diseases (e.g. insulin resistance, hyperlipidemia, atherosclerosis) and therefore do not support further investigation of the effect of specific therapy to increase concentrations of these adipokines (i.e. Thiazolidinediones, n3PUFAs) at this time. Nevertheless, the negative associations between adiponectin and triglyceride and insulin in healthy dogs and cats, support a hypolipidemic and insulin-sensitizing effect of this adipokine. These findings warrant further investigation into the role of adiponectin in the prevention of the development of overt DM or HL in healthy obese at risk cats, or prevention of complications in hypothyroid or diabetic dogs, by modulation of secretion.

The diets of all dogs and cats in these studies were not controlled. All healthy dogs and cats were fed nutritionally completed, commercially available diets with no dietary supplements or treats. On the other hand, complete dietary information was not available for some of the dogs and cats enrolled in the study groups of obesity-related diseases and a few may have been supplemented or fed specific diets. This could have contributed to differences in n3PUFA

concentrations between study and control groups in the studies investigating obesity-related diseases. This may be of special importance in cats with HL, since although most cats were presented with anorexia of several days to weeks, a few may have been encouraged to eat by the owners using fish products, that would increase their adipose tissue content of n3PUFA and subsequently result in higher serum n3PUFAs concentrations following adipose tissue lipolysis. Marked differences in dietary n3PUFAs content are unlikely between the healthy dogs and cats and those with the other diseases investigated (canine hypothyroidism, canine and feline DM) since the population from which dogs and cats were selected for the study and control groups was homogenic, and the diets are assumed to have been similar; nevertheless, differences in the diets cannot be ruled out as dietary analysis could not be completed in these dogs with naturally-occurring diseases.

Associations revealed with n3PUFAs in some obesity-related diseases in these studies suggest potential beneficial effects of these FAs. The negative associations with liver enzymes in cats with HL and DM suggest a hepatoprotective effect, and the negative association with triglyceride in cats with HL supports a hypolipidemic effect. These findings warrant further investigation into the role of dietary supplementation with n3PUFA as adjunct therapy in obesity-related diseases in cats.

APPENDICES

A. Assay interference testing

Study aims

- Determine effects of hemolysis, lipemia, and bilirubinemia on measurements of concentrations of adiponectin, leptin, TNF α , and IL6 in canine and feline serum.

Methods

Serum samples

Two pools of canine or feline serum were used for each assay tested. Each pool was divided into 17 aliquotes. Phosphate Buffer Solution (PBS), chloroform, or 5 different dilutions of hemolysate, lyposyn, or bilirubin solution were added to each aliquot (See Appendix C, Protocol 5). Final concentrations of the interfering substances were as follows: hemoglobin in dog serum: 0.0, 0.1, 0.4, 1.7, 6.6, 26.4 $\mu\text{g}/\mu\text{L}$; hemoglobin in cat serum: 0.0, 0.1, 0.4, 1.8, 7.1, 28.3 $\mu\text{g}/\mu\text{L}$; lipid: 0, 6, 25, 100, 400, 1600 mg/dL; bilirubin: 0.0, 0.2, 0.8, 3.1, 12.5, 50.0 mg/dL.

Assays

Serum samples from dogs were used for measurements of leptin (Canine Leptin ELISA, Millipore), adiponectin (Canine Adiponectin ELISA Millipore), insulin (Human Insulin RIA, Diagnostic Systems Laboratories), and IL6 (Canine IL6 ELISA, R&D Systems). Serum samples from cats were used for measurements of leptin (Multispecies Leptin RIA kit, Millipore), adiponectin (Mouse/rat Adiponectin ELISA kit, B-Bridg), and TNF α (Feline TNF α ELISA,

R&D Systems). For assays details see Appendix C, Protocols 1 and 2. All samples from each pool were run in one assay.

Results

Table 33 presents the minimum and maximum percent of the expected value for the measured values with variable concentrations of interfering substances and the CV of all measurements for each pool of serum. All the CV results, but those the Canine IL6 ELISA assay, were within the acceptable range (<15%). The high CV in this assay may be the result of the relatively low measured concentrations of IL6 in this pool of serum. Figures 22 and 23 illustrate the measured concentrations of analytes with different concentrations of interfering substances. Measured concentrations of one of the two pools were above the assay's dynamic range for Canine IL6 and Feline TNF α , therefore results are only available for one pool for these assays.

Conclusions

Concentrations of up to about 25 $\mu\text{g}/\mu\text{L}$ hemoglobin, 1600 mg/dL lipid, and 50 mg/dL bilirubin did not interfere in the examined assays for measurement of leptin, adiponectin, and insulin in dogs and cats. Some interference may occur with the examined assays for measurements IL6 and TNF α .

Table 33. Effect of hemoglobin, lipid, and bilirubin on measured concentrations of leptin, adiponectin, insulin, IL6 and TNF α in serum pools from dogs and cats.

Assay	Interfering substance	Percent of expected value range (%)†		CV (%)‡	
		Pool 1	Pool 2	Pool 1	Pool 2
Canine Leptin ELISA	Hemoglobin	98-118	95-105	8.9	3.7
	Lipid	88-98	102-116	4.2	5.5
	Bilirubin	112-133	98-132	9.6	12.3
Mouse/rat Adiponectin ELISA	Hemoglobin	93-124	86-108	9.7	8.2
	Lipid	106-119	102-122	6.1	7.8
	Bilirubin	91-104	88-121	4.9	11.3
Canine Adiponectin ELISA	Hemoglobin	85-100	95-102	6.6	2.5
	Lipid	88-99	96-106	5.4	4.0
	Bilirubin	96-110	95-99	5.6	2.2
Multispecies Leptin RIA	Hemoglobin	99-123	93-106	8.3	4.8
	Lipid	104-115	96-110	4.6	5.7
	Bilirubin	110-112	89-104	4.4	6.0
Human Insulin RIA	Hemoglobin	85-93	82-101	5.8	7.8
	Lipid	95-116	99-113	7.0	5.2
	Bilirubin	92-102	96-101	4.3	1.8
Canine IL6 ELISA	Hemoglobin	111-381	-	61.5	-
	Lipid	116-177	-	22.8	-
	Bilirubin	109-157	-	17.5	-
Feline TNF α ELISA	Hemoglobin	104-115	-	6.6	-
	Lipid	75-95	-	12.2	-
	Bilirubin	95-125	-	12.2	-

† Expected value is the measured concentration in a serum sample with added solvent (PBS for hemoglobin and lipid, chloroform for bilirubin).

‡ Calculated from all measured concentrations for each pool.

Figure 24. Measured concentrations of leptin, adiponectin, insulin, and IL6 in two pools of canine serum with added hemoglobin, lipid, or bilirubin in variable concentrations.

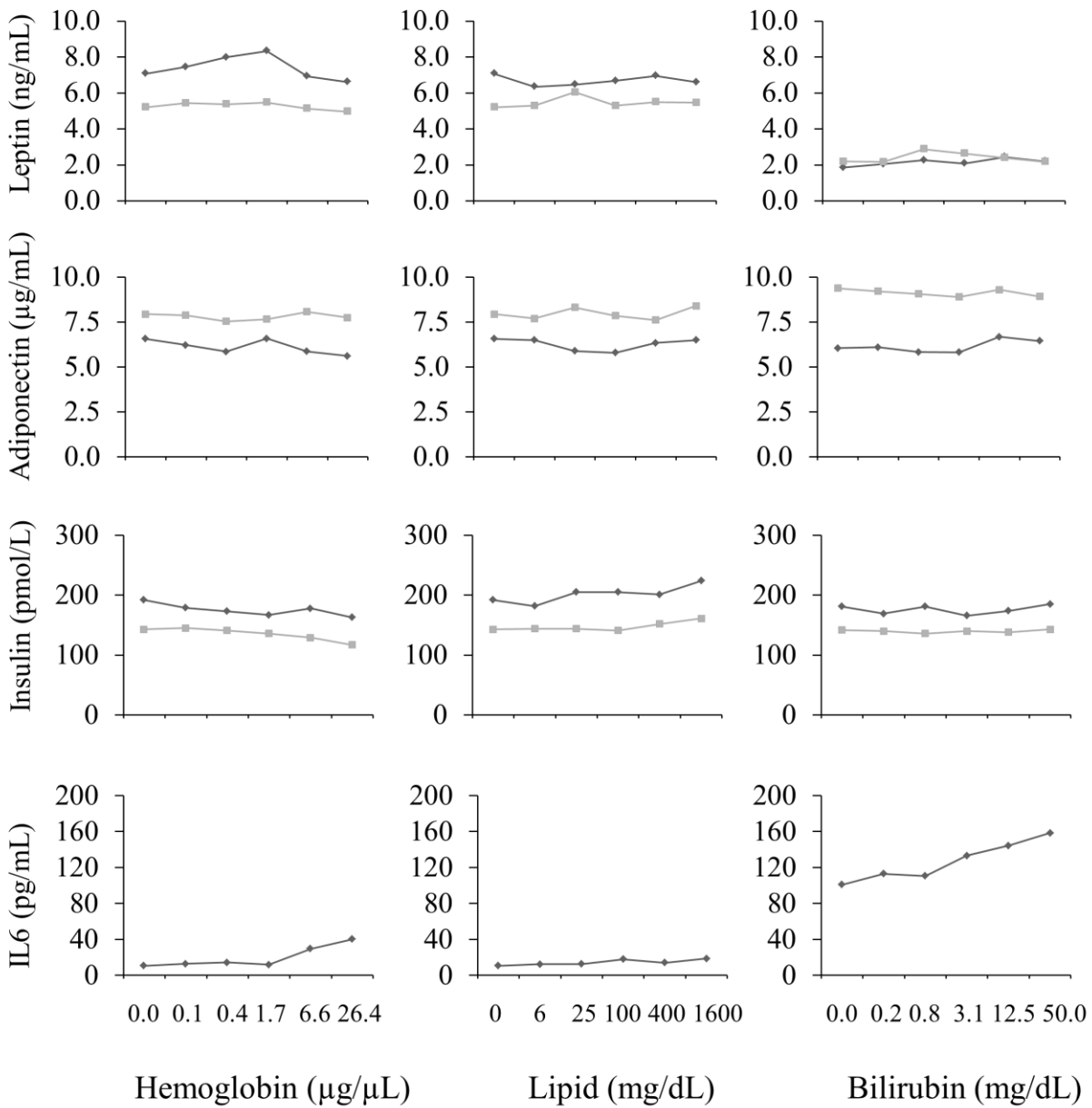
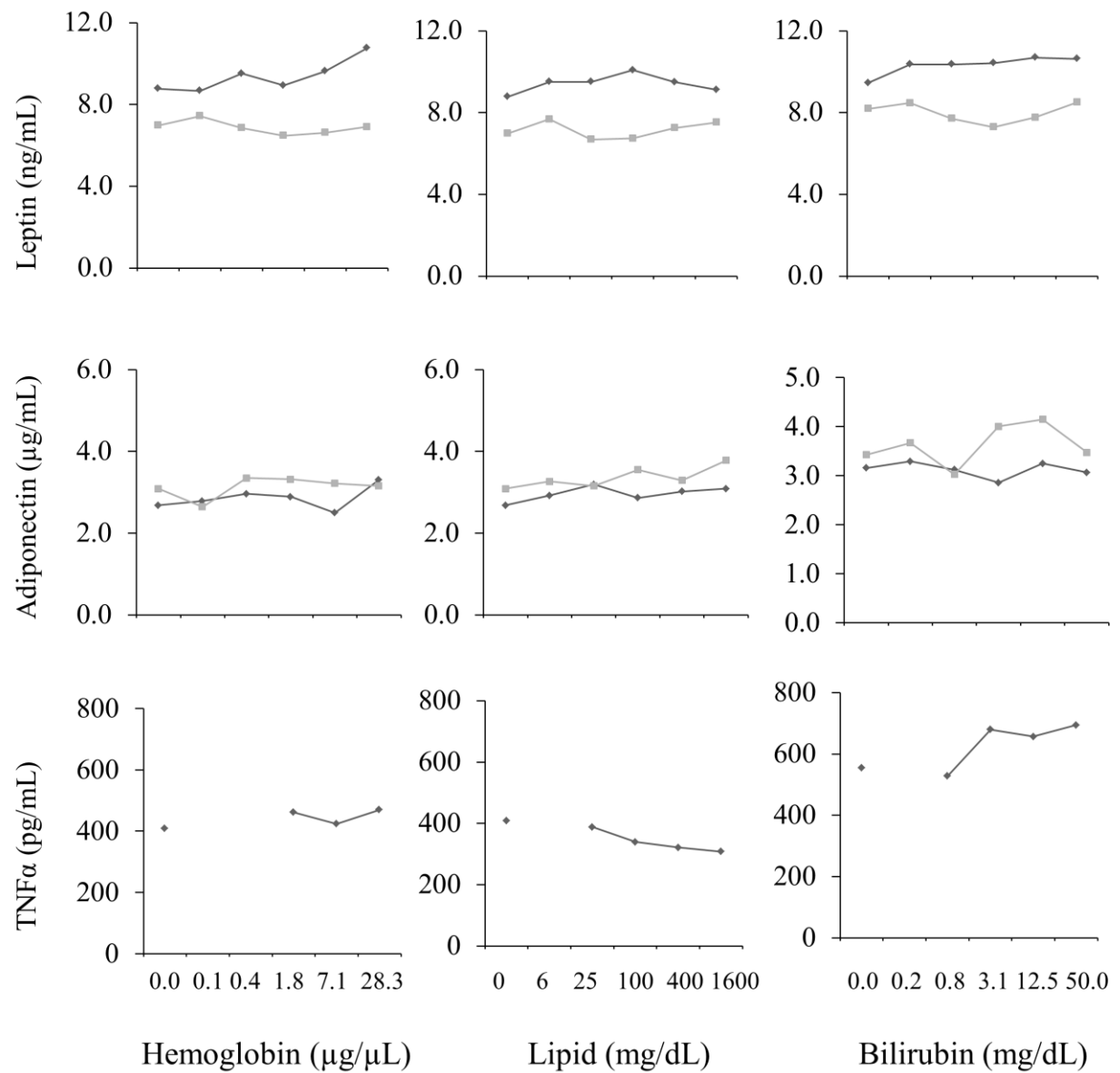


Figure 25. Measured concentrations of leptin, adiponectin, and TNF α in two pools of feline serum with added hemoglobin, lipid, or bilirubin in variable concentrations.



B. Stability of long chain fatty acids in fish oil gel capsules

Study aims

- Compare FA composition of fish oil gel capsule.

Methods

FA analysis of a fish oil gel capsule (Enteric Coated Omega 3 Fish Oil softgel, Spring Valley) content (See Appendix C, Protocol 3) was determined immediately after unsealing the original bottle and after 60 days of storing in an open bottle at room temperature.

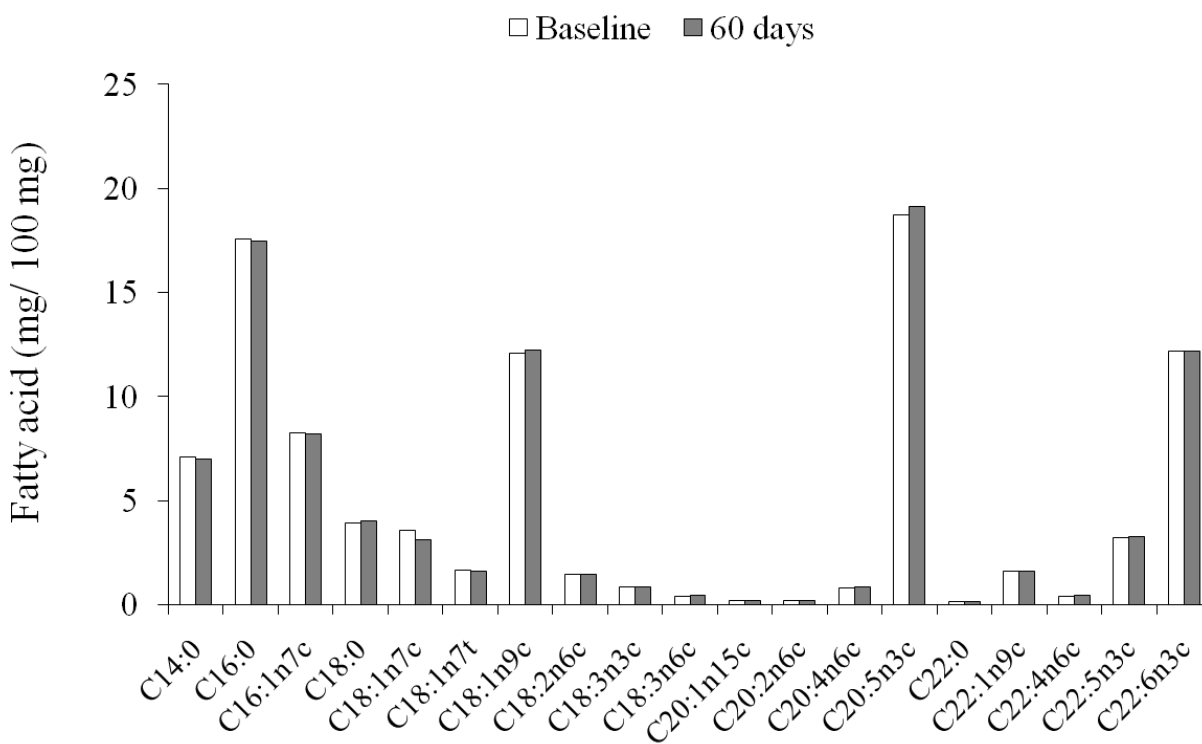
Results

FA composition is presented in Figure 24.

Conclusion

No differences between FA composition at baseline and at 60 days were detected.

Figure 26. FA composition of a fish oil gel capsule at baseline and after 60 days at room temperature.



C. Procedures protocols

1. ELISAs

Canine Adiponectin ELISA kit (Millipore EZCADP63K)

Reagents

1. Canine Adiponectin ELISA Plate - Coated with a Monoclonal Anti-Adiponectin Capture Antibody.
2. 10X Concentrate HRP Wash Buffer- 50mM Tris Buffered Saline containing Tween-20.
3. Canine Adiponectin Calibrator (Standard)- Purified Recombinant Canine Adiponectin, lyophilized.
4. Quality Control 1 and 2- containing adiponectin in assay buffer at two different levels, lyophilized.
5. Assay Buffer - 0.05M PBS pH 7.4 containing 0.025M EDTA, 0.08% Sodium Azide, 1% BSA.
6. Canine Adiponectin Detection Antibody - Pre-titered Biotinylated Anti-Canine Adiponectin Polyclonal Antibody.
7. Enzyme Solution -Pre-titered Streptavidin-HRP Conjugate in Buffer.
8. Substrate (Light sensitive, avoid unnecessary exposure to light) - TMB in Assay Buffer
9. Stop Solution- 0.3 M HCl

Allow all reagents and serum samples to come to room temperature.

Sample Preparation:

1. Dilute serum 1:2,000 in Assay Buffer
2. Culture media dilution depends on the study design (in the present studies medium samples were run undiluted).

Canine Adiponectin Standard (Calibrator) Preparation

1. Reconstitute the canine adiponectin standard with 0.5ml deionized water to give a concentration of 100 mg/mL. Invert and mix gently, allow the vial to set for 5 minutes, mix again.
2. Prepare a serial 1:3 dilution of the standard:
 - a. Transfer 0.2 mL of the reconstituted standard (100 ng/mL) to another tube containing 0.4 mL assay buffer, mix well.
 - b. Transfer 0.2 mL of the previous dilution to another tube containing 0.4 mL assay buffer, mix well
 - c. Repeat step 2.b. 4 more times.

Canine Adiponectin Quality Control Preparation

1. Reconstitute each of the Canine Adiponectin Quality Control 1 and Quality Control 2 vials with 0.5 mL of deionized water. Invert gently to mix, let set for 5 minutes, mix well.

Assay Procedure

1. Dilute the 10X Wash Buffer concentrate 10 fold by mixing the 100 mL HRP Wash Buffer with 900 mL deionized or distilled water.
2. Wash the plate 3 times with 300µL of diluted 1X Wash Buffer. Decant Wash Buffer from all wells by inverting the plate and tapping firmly onto absorbent towels several times. Do not let wells dry before proceeding to the next step.
3. Add 50 µL Assay Buffer to all wells.
4. Add an additional 50 µL Assay Buffer to the duplicate blank wells.
5. Add 50 µL Canine Adiponectin Standard in duplicate to the appropriate wells standards, pipetting in ascending order of concentration. Add in duplicate 50 µL QC1 and QC2 to the appropriate wells for Quality Controls. Add sequentially 50 µL of diluted serum samples or undiluted medium samples in duplicate to the remaining wells for samples (Figure 27).

Figure 27. Canine Adiponectin ELISA plate arrangement.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	St 4	QC1	Spl 3	Spl 7	Spl 11	Spl 15	Spl 19	Spl 23	Spl 27	Spl 31	Spl 35
B	"	3.7	"	"	"	"	"	"	"	"	"	"
C	St 1	St 5	QC2	Spl 4	Spl 8	Spl 12	Spl 16	Spl 20	Spl 24	Spl 28	Spl 32	Spl 36
D	0.14	11.11	"	"	"	"	"	"	"	"	"	"
E	St 2	St 6	Spl 1	Spl 5	Spl 9	Spl 13	Spl 17	Spl 21	Spl 25	Spl 29	Spl 33	QC1
F	0.41	33.33	"	"	"	"	"	"	"	"	"	"
G	St 3	St 7	Spl 2	Spl 6	Spl 10	Spl 14	Spl 18	Spl 22	Spl 26	Spl 30	Spl 34	QC2
H	1.23	100	"	"	"	"	"	"	"	"	"	"

6. Cover plate with sealer and incubate at room temperature for 2.0 hours with shaking on a microtiter plate shaker set at approximately 400 to 500 rpm.
7. Remove the plate sealer and decant solutions from each well. Tap as before to remove residual solutions from the plate.

8. Wash plate 3 times with 300 μ L 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer
9. Add 100 μ L Detection Antibody to all wells. Cover the plate with a plate sealer and incubate at room temperature for 1.0 hour with shaking on a microtiter plate shaker set at approximately 400 to 500 rpm.
10. Remove the plate sealer and decant solutions from each well. Tap as before to remove residual solutions from the plate.
11. Wash plate 3 times with 300 μ L 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer.
12. Add 100 μ L Enzyme Solution to each well. Cover plate with a sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker with the same setting.
13. Remove sealer, decant solutions from the plate, and tap plate to remove the residual fluid.
14. Wash plate 3 times with 300 μ L 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer.
15. Add 100 μ L of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker with the same setting for 5 - 20 minutes. Blue color should be formed in wells of Adiponectin standards with intensity proportional to increasing concentrations of Adiponectin.
16. Remove sealer and add 100 μ L Stop Solution, shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification.
17. Read absorbance at 450 nm and 590 nm (potential) in a plate reader within 5 minutes and ensure that there are no air bubbles in any well.

18. Record the difference of absorbance units. The absorbance of highest Adiponectin standard should be approximately 2.6-2.8 or not to exceed the capability of the plate reader used.

The assay will be considered acceptable when all Quality Control values fall within the calculated Quality Control Range.

If the difference between duplicate results of a sample is >15% CV, repeat the sample.

The limit of sensitivity of this assay is approximately 0.05 ng/mL Canine Adiponectin.

The approximate dynamic range of this assay is 0.137 - 100 ng/mL Canine Adiponectin in a 50 μ L sample size.

Intra- and inter-assay CVs are 4% and 8%.

Mouse/rat Adiponectin ELISA kit (B-Bridge K1002-1)

Reagents

1. Adiponectin ELISA Plate - Coated with rabbit anti-mouse adiponectin polyclonal antibody.
2. 25X Concentrate HRP Wash Buffer- 50mM Tris Buffered Saline containing Tween-20.
3. Adiponectin Calibrator (Standard)- Purified Recombinant mouse adiponectin (8.0 ng/mL).
4. 5X Sample diluent.
5. Adiponectin Detection Antibody – Pre-titered Biotinylated rabbit anti-mouse adiponectin polyclonal antibody.

6. Enzyme -HRP Conjugate Streptavidin solution and streptavidin diluent.
7. Substrate A and substrate B.
8. Stop Solution- 0.3 M HCl

Allow all reagents and serum samples to come to room temperature.

Sample Preparation:

1. Prepare 1X Sample Diluent by mixing 50 mL 5X Sample Diluent with 200 mL of deionized water.
2. Dilute serum samples 1:1,111 with sample diluent.
3. Culture media dilution depends on the study design (in the present studies medium samples were run undiluted).

Adiponectin Standard (Calibrator) Preparation

1. Prepare a serial 1:2 dilution of the standard:
 - a. Transfer 0.25 mL of the reconstituted standard (8.00 ng/mL) to another tube containing 0.25 mL assay buffer, mix well.
 - b. Transfer 0.25 mL of the previous dilution to another tube containing 0.25 mL assay buffer, mix well
 - c. Repeat step 2.b. 3 more times.

Assay Procedure

1. Dilute the 25X Wash Buffer concentrate 25 fold by mixing the 40 mL HRP Wash Buffer with 960 mL deionized water.
2. Wash the plate 3 times with 350µL of diluted 1X Wash Buffer. Decant Wash Buffer from all wells by inverting the plate and tapping firmly onto absorbent towels several times.
Do not let wells dry before proceeding to the next step.
3. Add 100 µL Assay Buffer to the duplicate blank wells.
4. Add 100 µL Adiponectin Standard in duplicate to the appropriate wells standards, pipetting in ascending order of concentration. Add sequentially 100 µL of dilutes serum samples or undiluted medium samples in duplicate to the remaining wells for samples (Figure 28).

Figure 28. Mouse/rat Adiponectin ELISA plate arrangement.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	St 4	Spl 2	Spl 6	Spl 10	Spl 14	Spl 18	Spl 22	Spl 26	Spl 30	Spl 34	Spl 38
B	"	2.00	"	"	"	"	"	"	"	"	"	"
C	St 1	St 5	Spl 3	Spl 7	Spl 11	Spl 15	Spl 19	Spl 23	Spl 27	Spl 31	Spl 35	Spl 39
D	0.25	4.00	"	"	"	"	"	"	"	"	"	"
E	St 2	St 6	Spl 4	Spl 8	Spl 12	Spl 16	Spl 20	Spl 24	Spl 28	Spl 32	Spl 36	Spl 40
F	0.50	8.00	"	"	"	"	"	"	"	"	"	"
G	St 3	Spl 1	Spl 5	Spl 9	Spl 13	Spl 17	Spl 21	Spl 25	Spl 29	Spl 33	Spl 37	Spl 41
H	1.00	"	"	"	"	"	"	"	"	"	"	"

5. Cover plate with sealer and incubate at room temperature for 1.0 hour.
6. Remove the plate sealer and decant solutions from each well. Tap as before to remove residual solutions from the plate.
7. Wash plate 3 times with 350 µL 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer.

8. Add 100 μ L Detection Antibody to all wells. Cover the plate with a plate sealer and incubate at room temperature for 1.0 hour.
9. Remove the plate sealer and decant solutions from each well. Tap as before to remove residual solutions from the plate.
10. Wash plate 3 times with 300 μ L 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer.
11. Prepare enzyme solution (HRP-Conjugated Streptavidin) by mixing 60 μ L of HRP-Conjugated Streptavidin and 12mL of Streptavidin Diluent.
12. Add 100 μ L Enzyme Solution to each well. Cover plate with a sealer and incubate with moderate shaking at room temperature for 1.0 hour.
13. Remove sealer, decant solutions from the plate, and tap plate to remove the residual fluid.
14. Wash plate 3 times with 350 μ L LX Wash Buffer. Decant and tap firmly after each wash to remove residual buffer.
15. Prepare substrate solution by mixing 6.0 mL Substrate A and 6.0 mL Substrate B.
16. Add 100 μ L of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker with the same setting for 15 minutes. Blue color should be formed in wells of Adiponectin standards with intensity proportional to increasing concentrations of Adiponectin.
17. Remove sealer and add 100 μ L Stop Solution, shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification.
18. Read absorbance at 450 nm and 650nm (potential) in a plate reader within 5 minutes and ensure that there are no air bubbles in any well.
19. Record the difference of absorbance units.

If the difference between duplicate results of a sample is >15% CV, repeat the sample.

The limit of sensitivity of this assay is approximately 15.6 pg/mL Adiponectin.

The approximate dynamic range of this assay is 0.25 – 8.0 ng/mL Adiponectin.

Intra- and inter-assay CVs are 5% and 7%.

Canine Leptin ELISA kit (Millipore EZCL-31K)

Reagents

1. Canine Leptin ELISA Plate - Coated with Goat anti-Canine Leptin Antibody.
2. 10X Concentrate HRP Wash Buffer- 50mM Tris Buffered Saline containing Tween-20.
3. Canine Leptin Calibrator (Standard)- Purified Recombinant Canine Leptin, lyophilized, lyophilized.
4. Quality Controls 1 and 2- containing leptin in assay buffer at two different levels, lyophilized.
5. Assay Buffer - 0.05M PBS pH 7.4 containing 0.025M EDTA, 0.08% Sodium Azide, 1% BSA.
6. Canine Leptin Detection Antibody - Pre-titered Biotinylated Goat Anti-Canine Leptin Antibody.
7. Enzyme Solution -Pre-titered Streptavidin-HRP Conjugate in Buffer.
8. Substrate (Light sensitive, avoid unnecessary exposure to light) - TMB in Assay Buffer
9. Stop Solution- 0.3 M HCl.

Allow all reagents and serum samples to come to room temperature.

Sample Preparation:

1. Serum samples are run undiluted.
2. Culture media dilution depends on the study design (in the present studies medium samples were run undiluted).

Canine Leptin Standard (Calibrator) Preparation

1. Reconstitute the canine leptin standard with 0.5ml deionized water to give a concentration of 50 mg/mL. Invert and mix gently, allow the vial to set for 5 minutes, mix again.
2. Prepare a serial 1:2 dilution of the standard:
 - a. Transfer 0.25 mL of the reconstituted standard (50 ng/mL) to another tube containing 0.25 mL assay buffer, mix well.
 - b. Transfer 0.25 mL of the previous dilution to another tube containing 0.25 mL assay buffer, mix well
 - c. Repeat step 2.b. 4 more times.

Canine leptin Quality Control Preparation

1. Reconstitute each of the Canine leptin Quality Control 1 and Quality Control 2 vials with 0.5 mL of deionized water. Invert gently to mix, let set for 5 minutes, mix well.

Assay Procedure

1. Dilute the 10X Wash Buffer concentrate 10 fold by mixing the 100 mL HRP Wash Buffer with 900 mL deionized or distilled water.
2. Wash the plate 3 times with 300µL of diluted 1X Wash Buffer. Decant Wash Buffer from all wells by inverting the plate and tapping firmly onto absorbent towels several times.
Do not let wells dry before proceeding to the next step.
3. Add 80 µL Assay Buffer to all wells.
4. Add an additional 20 µL Assay Buffer to the duplicate blank wells.
5. Add 20 µL Canine Leptin Standard in duplicate to the appropriate wells standards, pipetting in ascending order of concentration. Add in duplicate 20 µL QC1 and QC2 to the appropriate wells for Quality Controls. Add sequentially 20 µL of serum or medium samples in duplicate to the remaining wells for samples (Figure 29).

Figure 29. Canine Leptin ELISA arrangement.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	St 4	QC1	Spl 3	Spl 7	Spl 11	Spl 15	Spl 19	Spl 23	Spl 27	Spl 31	Spl 35
B	"	6.25	"	"	"	"	"	"	"	"	"	"
C	St 1	St 5	QC2	Spl 4	Spl 8	Spl 12	Spl 16	Spl 20	Spl 24	Spl 28	Spl 32	Spl 36
D	0.78	12.5	"	"	"	"	"	"	"	"	"	"
E	St 2	St 6	Spl 1	Spl 5	Spl 9	Spl 13	Spl 17	Spl 21	Spl 25	Spl 29	Spl 33	QC1
F	1.56	25	"	"	"	"	"	"	"	"	"	"
G	St 3	St 7	Spl 2	Spl 6	Spl 10	Spl 14	Spl 18	Spl 22	Spl 26	Spl 30	Spl 34	QC2
H	3.13	50	"	"	"	"	"	"	"	"	"	"

6. Cover plate with sealer and incubate at room temperature for 2.0 hours with shaking on a microtiter plate shaker set at approximately 400 to 500 rpm.
7. Remove the plate sealer and decant solutions from each well. Tap as before to remove residual solutions from the plate.

8. Wash plate 3 times with 300 μ L 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer.
9. Add 100 μ L Detection Antibody to all wells. Cover the plate with a plate sealer and incubate at room temperature for 1.0 hour with shaking on a microtiter plate shaker set at approximately 400 to 500 rpm.
10. Remove the plate sealer and decant solutions from each well. Tap as before to remove residual solutions from the plate.
11. Wash plate 3 times with 300 μ L 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer.
12. Add 100 μ L Enzyme Solution to each well. Cover plate with a sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker with the same setting.
13. Remove sealer, decant solutions from the plate, and tap plate to remove the residual fluid.
14. Wash plate 3 times with 300 μ L 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer.
15. Add 100 μ L of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker with the same setting for 5 - 20 minutes. Blue color should be formed in wells of Leptin standards with intensity proportional to increasing concentrations of Leptin.
16. Remove sealer and add 100 μ L Stop Solution, shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification.
17. Read absorbance at 450 nm and 590nm (potential) in a plate reader within 5 minutes and ensure that there are no air bubbles in any well.

18. Record the difference of absorbance units. The absorbance of highest leptin standard should be approximately 2.2-2.8 or not to exceed the capability of the plate reader used.

The assay will be considered acceptable when all Quality Control values fall within the calculated Quality Control Range.

If the difference between duplicate results of a sample is >15% CV, repeat the sample.

The limit of sensitivity of this assay is approximately 0.4 ng/mL Canine Leptin.

The approximate dynamic range of this assay is 0.78 - 100 ng/mL Canine Leptin in a 20 µL sample size.

Intra- and inter-assay CVs are 4% and 8%.

Canine IL6 ELISA, Feline IL6 ELISA, Canine TNFα ELISA, and Feline TNFα ELISA Duosets (R&D Systems DY1609, DY1507, DY2305, DY2586)

Reagents and materials for all duo sets

1. PBS- 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2 - 7.4, 0.2 µm filtered.
2. Reagent diluent- 1% BSA – dilute 1:10 of RD (10% BSA) in demonized water. pH – 7.2-7.4, 0.2 um filtered.
3. Wash Buffer- 0.05% Tween 20 – dilute 1:25 in demonized water.
4. Streptavidin-HRP- 1.0 mL of streptavidin conjugated HRP. Dilute 1:200 to a working concentration.
5. Substrate Solution- mix 6 mL of color reagent A with 6 mL of color reagent B.

6. Stop Solution - 2 N H₂SO₄.
7. 96-well microplate.

Reagents for canine IL6 assay

1. Capture Antibody - 144 µg/mL of goat anti-canine IL6 when reconstituted with 1.0 mL of PBS. Dilute to a working concentration of 0.8 µg/mL in PBS.
2. Detection Antibody - 36 µg/mL of biotinylated goat anti-canine IL6 when reconstituted with 1.0 mL of Reagent Diluent. Dilute to a working concentration of 200 ng/mL in Reagent Diluent.
3. Standard - Each vial contains 100 ng/mL of recombinant canine IL6 when reconstituted with 0.5 mL of Reagent Diluent. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Make a seven point standard curve using 2-fold serial dilutions in Reagent Diluent, and a high standard of 4000 pg/mL.
4. Controls- reconstitute 25 µg recombinant canine IL6 in 225 µL PBS to get 100 µg/mL. Make 2 different dilutions to get QC1-100 pg/mL and QC2- 500 pg/mL.

Reagents for feline IL6 assay

1. Capture Antibody – 360 µg/mL of mouse anti-feline IL6 when reconstituted with 1.0 mL of PBS. Dilute to a working concentration of 2.0 µg/mL in PBS.
2. Detection Antibody - 72 µg/mL of biotinylated goat anti-feline IL6 when reconstituted with 1.0 mL of Reagent Diluent. Dilute to a working concentration of 400 ng/mL in Reagent Diluent.

3. Standard - Each vial contains 100 ng/mL of recombinant feline IL6 when reconstituted with 0.5 mL of Reagent Diluent. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Make a seven point standard curve using 2-fold serial dilutions in Reagent Diluent, and a high standard of 2000 pg/mL.
4. Controls- reconstitute 25 µg recombinant feline IL6 in 225 µL PBS to get 100 µg/mL. Make 2 different dilutions to get QC1-100 pg/mL and QC2- 500 pg/mL.

Reagents for canine TNFα assay

1. Capture Antibody – 180 µg/mL of mouse anti-canine TNFα when reconstituted with 1.0 mL of PBS. Dilute to a working concentration of 1.0 µg/mL in PBS.
2. Detection Antibody - 18 µg/mL of biotinylated goat anti-canine TNFα when reconstituted with 1.0 mL of Reagent Diluent. Dilute to a working concentration of 100 ng/mL in Reagent Diluent.
3. Standard - Each vial contains 100 ng/mL of recombinant canine TNFα when reconstituted with 0.5 mL of Reagent Diluent. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Make a seven point standard curve using 2-fold serial dilutions in Reagent Diluent, and a high standard of 1000 pg/mL.
4. Controls- reconstitute 25 µg recombinant canine TNFα in 225 µL PBS to get 100 µg/mL. Make 2 different dilutions to get QC1-100 pg/mL and QC2- 500 pg/mL.

Reagents for feline TNFα assay

1. Capture Antibody – 144 µg/mL of goat anti-feline TNFα when reconstituted with 1.0 mL of PBS. Dilute to a working concentration of 0.8 µg/mL in PBS.
2. Detection Antibody - 36 µg/mL of biotinylated goat anti-feline TNFα when reconstituted with 1.0 mL of Reagent Diluent. Dilute to a working concentration of 200 ng/mL in Reagent Diluent.
3. Standard - Each vial contains 90 ng/mL of recombinant feline TNFα when reconstituted with 0.5 mL of Reagent Diluent. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Make a seven point standard curve using 2-fold serial dilutions in Reagent Diluent, and a high standard of 1000 pg/mL.
4. Controls- reconstitute 25 µg recombinant feline TNFα in 225 µL PBS to get 100 µg/mL. Make 2 different dilutions to get QC1-100 pg/mL and QC2- 500 pg/mL.

Plate Preparation (all duosets)

1. Dilute the Capture Antibody to the working concentration in PBS. Immediately coat the plate with 100 µL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Wash plate 3 times with 400 µL 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer washes. Wash by filling each well with Wash Buffer.
3. Block plates by adding 300 µL of Reagent Diluent to each well. Incubate at room temperature for a minimum of 1 hour.
4. Wash plate 3 times with 400 µL 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer washes.

Assay Procedure (all duosets)

1. Add 100 μ L of standards, control, or sample per well in duplicate. Cover with an adhesive strip and incubate 2 hours at room temperature (Figures 30-33).

Figure 30. Canine IL6 ELISA plate arrangement.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	St4	QC1	Spl 3	Spl 7	Spl 11	Spl 15	Spl 19	Spl 23	Spl 27	Spl 31	Spl 35
B	"	500	"	"	"	"	"	"	"	"	"	"
C	St1	St5	QC2	Spl 4	Spl 8	Spl 12	Spl 16	Spl 20	Spl 24	Spl 28	Spl 32	Spl 36
D	62.5	1000	"	"	"	"	"	"	"	"	"	"
E	St2	St6	Spl 1	Spl 5	Spl 9	Spl 13	Spl 17	Spl 21	Spl 25	Spl 29	Spl 33	QC1
F	125	2000	"	"	"	"	"	"	"	"	"	"
G	St3	St7	Spl 2	Spl 6	Spl 10	Spl 14	Spl 18	Spl 22	Spl 26	Spl 30	Spl 34	QC2
H	250	4000	"	"	"	"	"	"	"	"	"	"

Figure 31. Feline IL6 ELISA plate arrangement.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	St4	QC1	Spl 3	Spl 7	Spl 11	Spl 15	Spl 19	Spl 23	Spl 27	Spl 31	Spl 35
B	"	250	"	"	"	"	"	"	"	"	"	"
C	St1	St5	QC2	Spl 4	Spl 8	Spl 12	Spl 16	Spl 20	Spl 24	Spl 28	Spl 32	Spl 36
D	31.3	500	"	"	"	"	"	"	"	"	"	"
E	St2	St6	Spl 1	Spl 5	Spl 9	Spl 13	Spl 17	Spl 21	Spl 25	Spl 29	Spl 33	QC1
F	62.5	1000	"	"	"	"	"	"	"	"	"	"
G	St3	St7	Spl 2	Spl 6	Spl 10	Spl 14	Spl 18	Spl 22	Spl 26	Spl 30	Spl 34	QC2
H	125	2000	"	"	"	"	"	"	"	"	"	"

Figure 32. Canine TNF α ELISA plate arrangement.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	St4	QC1	Spl 3	Spl 7	Spl 11	Spl 15	Spl 19	Spl 23	Spl 27	Spl 31	Spl 35
B	"	125	"	"	"	"	"	"	"	"	"	"
C	St1	St5	QC2	Spl 4	Spl 8	Spl 12	Spl 16	Spl 20	Spl 24	Spl 28	Spl 32	Spl 36
D	15.6	250	"	"	"	"	"	"	"	"	"	"
E	St2	St6	Spl 1	Spl 5	Spl 9	Spl 13	Spl 17	Spl 21	Spl 25	Spl 29	Spl 33	QC1
F	31.3	500	"	"	"	"	"	"	"	"	"	"
G	St3	St7	Spl 2	Spl 6	Spl 10	Spl 14	Spl 18	Spl 22	Spl 26	Spl 30	Spl 34	QC2
H	62.5	1000	"	"	"	"	"	"	"	"	"	"

Figure 33. Feline TNF α ELISA plate arrangement.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	St4	QC1	Spl 3	Spl 7	Spl 11	Spl 15	Spl 19	Spl 23	Spl 27	Spl 31	Spl 35
B	"	125	"	"	"	"	"	"	"	"	"	"
C	St1	St5	QC2	Spl 4	Spl 8	Spl 12	Spl 16	Spl 20	Spl 24	Spl 28	Spl 32	Spl 36
D	15.6	250	"	"	"	"	"	"	"	"	"	"
E	St2	St6	Spl 1	Spl 5	Spl 9	Spl 13	Spl 17	Spl 21	Spl 25	Spl 29	Spl 33	QC1
F	31.3	500	"	"	"	"	"	"	"	"	"	"
G	St3	St7	Spl 2	Spl 6	Spl 10	Spl 14	Spl 18	Spl 22	Spl 26	Spl 30	Spl 34	QC2
H	62.5	1000	"	"	"	"	"	"	"	"	"	"

2. Wash plate 3 times with 400 μ L 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer washes.
3. Add 100 μ L of the Detection Antibody, diluted in Reagent Diluent, to each well. Cover with an adhesive strip and incubate 2 hours at room temperature.
4. Wash plate 3 times with 400 μ L 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer washes.
5. Add 100 μ L of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

6. Wash plate 3 times with 400 μ L 1X Wash Buffer. Decant and tap firmly after each wash to remove residual buffer washes.
7. Add 100 μ L of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 μ L of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm and 540 nm or 570 nm. Subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate.

If the difference between duplicate results of a sample is >15% CV, repeat the sample.

Medium samples were diluted 1:100 in preparation for the canine IL6 and feline IL6 assays.

The approximate dynamic ranges are 6.1-4000 pg/mL for canine IL6 assay, 6.1-2000 pg/mL for feline IL6 assay, 2.4-1000 pg/mL for canine TNF α assay and feline TNF α assay.

Intra- and inter-assay CVs are 3% and 7% for the canine IL6 assay, the feline IL6 assay, and feline TNF α assay, and 8% and 10% for canine TNF α assay.

Calculations for ELISAs

1. Average duplicate absorbance for the blank, each standard, control, and sample.
2. Subtract the average blank absorbance from each average reading.
3. Create a standard curve by generating a four parameter logistic curve-fit.
4. Determine the concentration of hormone in unknown samples and controls by interpolation of the reference curve.

2. RIAs

Human Insulin RIA (Diagnostic Systems Laboratories DSL-1600)

Reagents

1. Insulin standards- one vial (B0) containing 0 pmol/L and five vials (A-E) containing 36, 108, 359, 1076, and 2153 pmol/L of insulin in a protein-based buffer, lyophilized.
Reconstitute standard B0 with 5mL of deionized water and Standards A-E with 1 mL deionized water.
2. ¹²⁵I-Insulin Reagent- One vial containing < 5μCi of [I-125]-labeled insulin in a protein-based buffer, lyophilized. Reconstitute with 11 mL deionized water.
3. Insulin antiserum- Guinea Pig anti-insulin serum, lyophilized. Reconstitute with 10 mL deionized water.
4. Precipitating Reagent- Goat anti-Guinea Pig IgG Serum with PEG and sodium azide, 105 mL.
5. Quality Controls 1 & 2- Purified Recombinant Human insulin, lyophilized. Reconstitute each with 1 mL deionized water.

Assay Procedure

A. Day 1:

1. Pipet 200 μ L of Standard B0 to the Non-Specific Binding (NSB) tubes (3-4), 100 μ L to Standard B0 the Reference (B0) tubes (5-6), and 100 μ L of standards A-E to tubes 7 through 16.
2. Pipet 100 μ L of Quality Controls in duplicate, pipet 100 μ L of each sample in duplicate (Figure 34).

Figure 34. Human Insulin RIA tubes arrangement.

1,2	TC	
3,4	NSB	
5,6	B0	0 pmol/L
7,8	St A	36 pmol/L
9,10	St B	108 pmol/L
11,12	St C	359 pmol/L
13,14	St D	1076 pmol/L
15,16	St E	2153 pmol/L
17,18	OC1	
19,20	QC2	
21-n	Spl	

3. Pipet 100 μ L of insulin antiserum to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
4. Pipet 100 μ L of 125 I-Human Leptin to all tubes.
5. Vortex, cover, and incubate overnight (16 hours) at 4°C.

B. Day 2:

1. Add 1.0 mL of cold (4°C) Precipitating Reagent to all tubes except Total Count tubes (1-2).
2. Vortex and incubate 10-15 minutes at room temperature (~25°C).
3. Centrifuge, 4°C, all tubes [except Total Count tubes (1-2)] for 20 minutes at 1,500 xg.

4. Immediately decant the supernate from all tubes except Total Count tubes (1-2), drain tubes for 15- 60 seconds (be consistent between racks), and blot excess liquid from lip of tubes.
5. Count all tubes in a gamma counter for 1 minute.

The assay will be considered accepted when all Quality Control values fall within the calculated Quality Control Range.

If the difference between duplicate results of a sample is >10% CV, repeat the sample.

The limit of sensitivity for the Insulin assay is 9 pmol/L (100 µL sample size).

The dynamic range of the assay is 9- 2153 pmol/L

Intra- and inter-assay CVs are 9% and 10%

Multispecies Leptin RIA kit (Millipore XL-85K)

Reagents

1. Assay Buffer- 0.05M Phosphosaline, pH 7.4, containing 0.025M EDTA, 0.08% Sodium Azide, 0.05% Triton X-100, and 1% RIA Grade BSA, 40 mL.
2. Antiserum- Guinea Pig anti-Multi-Species Leptin Antibody in Assay Buffer, 26 mL.
3. Assay Buffer containing Normal Guinea Pig IgG as a carrier, 27 mL.
4. 125I-Human Leptin - 125I-Human Leptin Label, HPLC purified, Lyophilized. Freshly iodinated label contains <3 µCi. Hydrate with 27 mL of Label Hydrating Buffer. Allow to set at room temperature for 30 minutes, with occasional gentle mixing.

5. Standards - Purified Recombinant Human Leptin in Assay Buffer at the following concentrations: 1, 2, 5, 10, 20, 50 ng/mL, 1mL each.
6. Quality Controls 1 & 2- Purified Recombinant Human Leptin in Assay Buffer. 1 mL each
7. Precipitating Reagent- Goat anti-Guinea Pig IgG Serum, 3% PEG and 0.05% Triton X-100 in 0.05M Phosphosaline, 0.025M EDTA, 0.08% Sodium Azide, 260 mL.

Assay Procedure

A., Day 1: Assay Set-Up

6. Pipet 300 μ L of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4), 200 μ L to the Reference (Bo) tubes (5-6), and 100 μ L of standards (St) A-F to tubes 7 through 18.
7. Pipet 100 μ L of Quality Controls in duplicate, pipet 100 μ L of each sample in duplicate (Figure 35).

Figure 35. Multispecies Leptin RIA tubes arrangement.

1,2	TC	
3,4	NSB	
5,6	B0	
7,8	St A	1 ng/mL
9,10	St B	2 ng/mL
11,12	St C	5 ng/mL
13,14	St D	10 ng/mL
15,16	St E	20 ng/mL
17,18	St F	50 ng/mL
19,20	OC1	
21,22	QC2	
23-n	Spl	

8. Pipet 100 μ L of Multi Species Leptin antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
9. Vortex, cover, and incubate overnight (20-24 hours) at 4°C.

B. Day 2:

10. Pipet 100 μ L of 125 I-Human Leptin to all tubes.
11. Vortex, cover, and incubate overnight (20-24 hours) at 4°C.

C. Day 3:

12. Add 1.0 mL of cold (4°C) Precipitating Reagent to all tubes except Total Count tubes (1-2).
13. Vortex and incubate 20 minutes at 4°C.
14. Centrifuge, 4°C, all tubes [except Total Count tubes (1-2)] for 20 minutes at 2,000-3,000 xg.
15. Immediately decant the supernate from all tubes except Total Count tubes (1-2), drain tubes for 15- 60 seconds (be consistent between racks), and blot excess liquid from lip of tubes.
16. Count all tubes in a gamma counter for 1 minute.

The assay will be considered accepted when all Quality Control values fall within the calculated Quality Control Range.

If the difference between duplicate results of a sample is >10% CV, repeat the sample.

The limit of sensitivity for the Multi Species Leptin assay is 1.0 ng/mL.

The dynamic range of the assays is 1-50 ng/mL.

Intra- and inter-assay CVs are 6% and 10%.

Calculations for RIAs

1. Average duplicate counts for Total Counts tubes (1-2), NSB tubes (3-4), Maximum Binding tubes (Reference, B0) (5-6), and all remaining tubes.
2. Subtract the average NSB counts from each average count (except for Total Counts).
3. Calculate the percentage of tracer bound: (Maximum Binding Counts/Total Counts) X 100. This should be 30-50%.
4. Calculate the percentage of maximum binding (%B/B0) for each standard and sample:
$$\%B/B0 = (\text{Sample or Standard/Maximum Binding}) \times 100.$$
5. Plot log(% B/B0) or logit(% B/B0) for each standard on the y-axis and log(known concentration of the standard) on the x-axis.
6. 6. Construct the reference curve by joining the points with a smooth curve.
7. Determine the concentration of hormone in unknown samples and controls by interpolation of the reference curve.

3. Total lipid determination and fatty acid analysis

Lipid extraction and determination of lipid content in serum samples

1. Pipette 400 μ L of serum sample into a 16x100 mm glass tube.
2. Add 600 μ L of 9% saline.
3. Add 1 mL ethanol (with BHT).
4. Add 1 mL hexane.
5. Cap and vortex for 10 min.
6. Centrifuge at 3000 rpm for 10 min.
7. Tare a 16x100 mm glass tube.
8. Using a positive displacement pipette, transfer 0.75 mL of the hexane (top) layer to the tarred tube.
9. Repeat steps 4,5,6, and 8 two more times.
10. Evaporate the hexane in the evaporator for 10 min.
11. Weigh the tube.
12. Calculate the total lipid mass.

Preparation of samples for FA analysis of fish oil gel capsules

Transfer 100 mg of the capsule content into a 16x100 mm glass tube.

Methylation of FAs (Sukhija and Palmquist, 1988)

1. Add 0.5 mL of hexane with internal standard (Heptadecanoic or Nonadecanoic acid 2 mg/mL) to all tubes (containing serum, lipid extracted from serum, or fish oil gel capsule content).
2. Mix briefly and wash the walls of the tube with the hexane.
3. Transfer the hexane to a screw-cap tube.
4. Calculate the lipid concentration of the lowest total lipid tube (for extracted lipid only).
5. Calculate the appropriate volume of hexane (without internal standard) needed to get the same lipid concentration in each of the other tubes. Add to the total lipid tubes, wash walls again and transfer to the appropriate screw-cap tube (for extracted lipid only).
6. Add 1 mL of 10% methanolic HCl to the tube.
7. Seal tube tightly with a Teflon-lined screw cap.
8. Mix briefly.
9. Heat tubes in 75 °C water bath for 1 hour.
10. Remove tubes from water bath and allow to cool.
11. If the hexane layer evaporated, add the same volume of hexane (without the internal standard).
12. Add 2 mL of 6% potassium carbonate, recap, and mix briefly.
13. Centrifuge at 1500 rpm for 5 min.
14. Place approximately 1 g of sodium sulfate in a 16x100 mm glass tube.
15. Transfer the hexane layer to the tube with sodium sulfate, mix using the pipette, transfer the hexane layer to a GC vial and seal.

Gas Chromatography

One μL samples of FA methyl esters in hexane are injected into the gas chromatograph (Clarus 500 Gas Chromatograph, Perkin Elmer). A fused silica capillary column (SP2560 Fused Silica Capillary column, Supelco -100m x 0.25mm with a 0.2 μm film thickness) is used to resolve the FA methyl esters in each sample. Hydrogen is used as a carrier gas at a flow of 0.5mL/min. The resolved components are detected by a flame ionization detector with an air flow of 400 mL/min and a hydrogen flow of 45 mL/min, the temperature of the detector is set at 255°C. Peaks are identified and validated using FA standards of known concentrations (Nu-check Prep, Elysian). Serum FA concentrations are reported as the percent concentrations.

4. Primary adipose tissue culture

Day 1:

Tissue preparation

1. Collect adipose tissue aseptically into a sterile 50 mL conical polypropylene tube containing cold medium (4 °C). Start procedure within 30 min.
2. Transfer the tissue into a sterile dish, rinse tissue with cold HBSS¹ (4 °C). Remove blood clots and excise blood vessels and connective tissue as possible.
3. Tar another sterile dish, transfer tissue, record weight.
4. Mince tissue to fragments about 0.5 cm size (1-5mg).
5. Rinse the tissue fragments with cold HBSS.
6. Add 1 mL medium² to 100 mg tissue fragments and incubate at room temperature for 1 hour. Then aspirate the medium.

Digestion

1. Transfer minced tissue to a tube containing warm (37 °C) collagenase³ solution (1mg/mL collagenase in medium), 2 mL collagenase solution for 1 g tissue fragments.
2. Tightly cap the tube and incubate at 37 °C in a shaking water bath / rotator for 1 hr.
3. Place a 100 µm sterile cell strainer on a sterile 50 mL conical tube.
4. Transfer the digested tissue into the sterile tube through the strainer.
5. Cap and centrifuge at 450Xg for 5 min.
6. Transfer the adipocyte layer to another sterile 50 mL conical tube.

Preparation of mature adipocytes culture:

1. Pipette out as much of the collagenase solution as possible from the tube with the adipocytes (from Step 6 of digestion) by inserting the pipette beneath the floating adipocytes layer.
2. Add warm medium to the tube with the adipocyte layer (same volume as collagenase solution) and spin at 450Xg for 5 min.
3. Pipette out as much of the medium as possible.
4. Repeat steps 2 and 3 one more time.
5. Add fresh medium (1mL per 100 mg adipocytes).
6. Cap (do not tighten completely) and incubate at 37 °C with 5% CO₂.

Preparation of SVCs culture:

1. Add RBC lysing buffer⁴ to the tube with the pellet (from Step 6 of digestion); 1 mL buffer for 1g digested tissue.
2. Shake gently to release the pellet, mix and let sit for 5 min.
3. Centrifuge at 450Xg for 5 min.
4. Decant the buffer and add fresh medium (same volume as collagenase solution).
5. Repeat steps 3 and 4 one more time.
6. Remove a small aliquot for determine no. of cells/mL by hemocytometer.
7. Divide suspension into 24-well plate. 1 mL in each well.
8. Cover and incubate at 37 °C with 5% CO₂.

Day 2:

Preparation of treatment media

1. Dilute FAs stock solution (50mM)⁵ in medium to make the necessary volume of each treatment medium (25μM, 50 μM, 100μM).
2. Add same volume of ethanol as volume of stock FA to medium to make control treatment.
3. Dilute troglitazone stock solution (20mg/mL)⁵ in medium to make the necessary volume of treatment medium (10μM).
4. Incubate diluted treatment media at 37 °C for 2 hours with occasional mixing before adding to cells.

Treatment- Mature adipocytes

1. Tar 5 mL polypropylene tubes.
2. Aspirate medium from tube containing mature adipocytes.
3. Transfer 100 μL aliquots of adipocytes into the 5 mL polypropylene tubes.
4. Weigh tubes with adipocytes and record adipocytes weight in each tube (as quickly as possible to avoid dehydration of cells).
5. Add 1 mL of different treatment medium to each tube.

Treatment- SVC

1. Aspirate medium from plate wells with SVC.
2. Add 1 mL of different treatment medium to each well (as quickly as possible to avoid dehydration of cells).

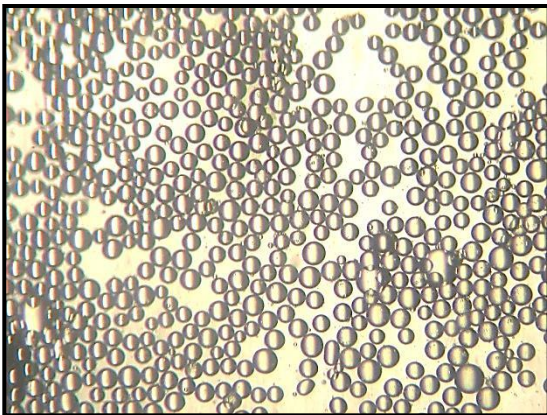
Day4: (following 48 hours of incubation in treatment medium)

Medium collection and determination of packed adipocytes volume (PAV) and SVC score

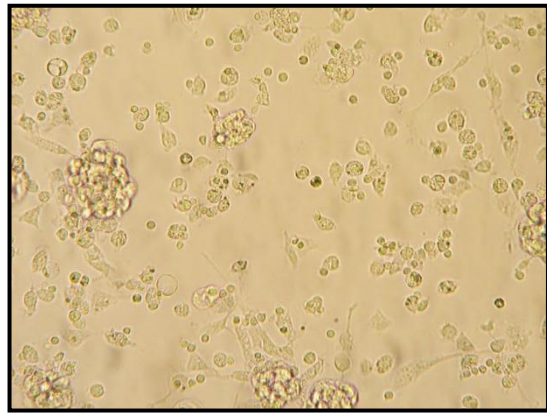
1. Aspirate a small aliquot of medium containing adipocytes from each treatment tube into a capillary hematocrit tube and centrifuge for 1 min in a Microhematocrit centrifuge. Measure the fractional occupation of the suspension by the adipocytes cells (packed adipocytes volume -PAV) (Honnor et al., 1985).
2. Examine culture plates microscopically and score wells according to the percentage of well area occupied by SVCs (SVC score; 1:10-20%, 2:21-40%, 3: 41-60%, 4:61-80, 5:81-100%). Exclude wells with less than 10% area occupation.
3. Aspirate medium from each tube or well and transfers into microcentrifuge tube.
4. Centrifuge the tubes for 10 min.
5. Transfer the supernatant into a clean tube and store at -80 °C until analysis.

Figure 36. Mature adipocytes (A) and SVC (B) from visceral fat of an intact female cat (X20)

A.



B.



For interpretation of the references to color in this figure, the reader is referred to the electronic version of this dissertation.

Solutions, supplements, and treatments

1) Table 34. Hanks' Balanced Salt Solution

(HBSS, Sigma H8265)

<i>Component</i>	<i>g/L</i>
CaCl ₂ •2H ₂ O	0.185
MgSO ₄ (anhyd)	0.09796
KCl	0.4
KH ₂ PO ₄ (anhyd)	0.06
NaHCO ₃	0.35
NaCl	8.0
Na ₂ HPO ₄ (anhydrous)	0.04788
D-Glucose	1.0

2) Medium with supplements-

Table 35. Dulbecco's Modified Eagle's Medium (DMEM)/Ham's Nutrient Mixture F-12

(Sigma D2906)

15.6 g powder dissolved in 1L of water.

<i>Inorganic Salts</i>	<i>g/L</i>
CaCl ₂ • 2H ₂ O	0.1166
CuSO ₄ • 5H ₂ O	0.0000013
Fe(NO ₃) ₃ • 9H ₂ O	0.00005
FeSO ₄ • 7H ₂ O	0.000417
MgCl ₂ • 6H ₂ O	0.0612
MgSO ₄	0.04884
KCl	0.3118
NaCl	6.996
Na ₂ HPO ₄	0.07102
NaH ₂ PO ₄	0.0543
ZnSO ₄ • 7H ₂ O	0.000432

Table 35 (cont'd)

<i>Vitamins</i>	<i>g/L</i>
D-Biotin	0.0000035
Choline Chloride	0.00898
Folic Acid	0.00266
myo-Inositol	0.0126
Niacinamide	0.00202
D-Pantothenic Acid • ½Ca	0.00224
Pyridoxal • HCl	0.002
Pyridoxine • HCl	0.000031
Riboflavin	0.000219
Thiamine • HCl	0.00217
Vitamin B12	0.00068

<i>Amino Acids</i>	<i>g/L</i>
L-Glutamine	0.365
Glycine	0.01875
L-Histidine • HCl • H ₂ O	0.03148
L-Isoleucine	0.05447
L-Leucine	0.05905
L-Lysine • HCl	0.09125
L-Methionine	0.01724
L-Phenylalanine	0.03548
L-Proline	0.01725
L-Serine	0.02625
L-Threonine	0.05345
L-Tryptophan	0.00902
L-Tyrosine • 2Na • 2H ₂ O	0.05579
L-Valine	0.05285

<i>Other</i>	<i>g/L</i>
D-Glucose	3.15
HEPES	3.5745
Hypoxanthine	0.0021
Linoleic Acid	0.000042
Putrescine • HCl	0.000081
Pyruvic Acid • Na	0.055
DL-Thioctic Acid	0.000105
Thymidine	0.000365

Table 36. Supplements added to medium.

<i>Supplement</i>	<i>Final concentration in medium</i>
HEPES	10 mM
BSA	10 mg/mL
Penicillin	100 U/mL
Streptomycin	0.1 mg/mL
Sodium bicarbonate	about 1.2 g/mL

- HEPES (Sigma H4034)- 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

- BSA (Sigma A8806)- FA free bovine serum albumin

-Penicillin and streptomycin (Sigma, P0781)

-Sodium bicarbonate- added to adjust the pH to 7.4

Filter sterilize.

Growth factors added to medium immediately prior to use-

Table 37. Preparation of growth factors stock solutions.

<i>Supplement</i>	<i>MW</i>	<i>Stock solution concentration (in water)</i>
Ethanolamine	61.08 (Density -1.012)	10 mg/mL
Sodium selenite	172.9	0.25 µg/mL
Ascorbic acid	176.12	10 mg/mL
Adenosine	267.2	50 µg/mL

- Ethanolamine (Sigma E0135)

-Sodium selenite (Sigma S5261)

- Ascorbic acid (Sigma, A4454)

-Adenosine (Sigma A4036)

Dissolve in water and filter sterilize; Store at -20 °C protected from light.

Table 38. Preparation of medium with growth factors.

<i>Supplement</i>	<i>Volume of stock solution added to 500 mL</i>	<i>Final concentration in medium</i>
Ethanolamine	250 µL	5 µg/mL
Sodium selenite	200 µL	0.1 ng/mL
Ascorbic acid	500 µL	55 µmol/L
Adenosine	500 µL	200 nmol/L

3) Collagenase solution-

Dissolve powder (Sigma C6885) in HBSS containing 1% BSA; 1 mg/mL

Filter sterilize.

Store frozen at -20 °C;

Thaw immediately prior to use.

Avoid more than 2 freeze/thaw cycles.

4) Red Blood Cells Lysing Buffer -

(Sigma R7757)

Ready to use.

5) Treatment stock solutions-

Table 39. Preparation of FAs stock solution.

	<i>MW</i>	<i>Density at 25C (g/mL)</i>	<i>Factor</i>
EPA	302.52	0.943	0.065051
AA	304.52	0.922	0.064593
PA	256.43	0.853	0.076822

Table 39 (cont'd)

EPA (Nu-Chek Prep, Inc. U-99-A)

AA (Nu-Chek Prep, Inc., U-71-A)

PA (Nu-Chek Prep, Inc., N-16-A)

Dissolve powder in absolute ethanol.

Volume of ethanol to add to make a 50mM solution=

$$= \text{Weight available} * [[1 / (\text{MW} * 0.05)] - [1 / (\text{Density} * 1000)]] =$$

$$= \text{Weight available} * \text{factor}$$

Filter sterilize.

Store in glass tubes at -20 °C, protect from light.

Troglitazone stock solution preparation

Dissolve 5 mg Troglitazone powder (Sigma T2573) in 0.25 mL DMSO (Sigma D2650) to make a 20 mg/mL solution.

(Due to small volume need to filter sterilize after dilution).

5. Preparation of serum samples for interference testing study

Hemolysate.

Preparation of hemolysate:

1. Obtain an EDTA blood sample (non-hemolyzed) with a known MCHC (Mean corpuscular hemoglobin concentration).
2. Centrifuge 5 min and remove plasma.
3. Add PBS to Red blood cells. Resuspend and centrifuge 5 min and remove PBS.
4. Repeat step 3 two more times.
5. Freeze the cells overnight to lyse the cells.

Dilution of hemolysate:

Make 1:4; 1:16; 1:64 and 1:256 dilutions of the hemolysate in PBS.

Serum samples preparation:

Add 40 μL of PBS, hemolysate and each dilution to 460 μL serum sample.

Lipid

Dilution of Liposyn 20% (contains 20 g/100 mL, equals to 200 $\mu\text{g}/\mu\text{L}$):

Make 1:4; 1:16; 1:64 and 1:256 dilutions of the Liposyn in PBS.

Serum samples preparation:

Add 40 μL of PBS, Liposyn and each dilution to 460 μL serum sample.

Bilirubin

Preparation of bilirubin solution:

Dissolve bilirubin powder in chloroform (10 mg/mL) (use a glass flask).

Dilution of bilirubin:

Make 1:4; 1:16; 1:64 and 1:256 dilutions of the bilirubin in chloroform (use glass tubes).

Serum samples preparation:

Add 25 μ L of chloroform, bilirubin and each dilution to 475 μ L serum sample.

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