# Development of new predictive biomarkers for early diagnosis of obesity in dogs and cats

**Gebin Li**

Dissertation submitted to Nippon Veterinary and Life Science

University in Partial Fulfillment of the Degree of Doctor of Philosophy

Under supervision of Professor Dr. Toshiro Arai

**May 2014**

# CONTENTS

# General Introduction

Overweight is caused by an excess energy intake, resulting in a positive energy balance and accumulation of lipid of adipose tissue. Excessive bodyweight (overweight and obesity) is a very common nutritional disorder in the small animal population, and is linked to both reduced overall lifespan (Kealy et al., 2002; Lawler et al., 2008) and a variety of secondary diseases (Markwell et al., 1990; German et al., 2006; Lund et al., 2006). A growing concern is the alarming increase in the prevalence of overweight dogs between 2006 (21%) and 2009 (35%) (People's Dispensary for Sick Animals., 2012), with half of all dogs in the UK currently being overweight (White et al., 2011). Moreover, a study in the Northeastern United States documented that 25% of 2000 cats presented to veterinarians were overweight (Scarlett et al., 1994).

Overweight and obese dogs, in particular, suffer from canine obesity-related metabolic dysfunctions (ORMD) (Tvarijonaviciute et al., 2012) which share components of metabolic syndrome in humans, such as hypoadiponectinaemia (Tvarijonaviciute et al., 2012), insulin resistance (German et al., 2009) hyperinsulinemia (Kim et al., 2003) and hypertriglyceridemia (Mori et al., 2011). Obese dogs can develop insulin resistance but not type 2 diabetes mellitus (Rand et al., 2004). Alternately, obesity is a significant risk factor for type 2 diabetes mellitus (T2DM), a common endocrinopathy in humans and in cats (Burkholder et al., 2000). Similar to humans, the increase in feline obesity can also be related to the following two main causes: 1) changes occurring in diet, comprising of excessive intake of

high-fat and carbohydrate foods, and 2) a lack of or reduced level of physical activity (Backus et al., 2007). As such, cats have recently been proposed as a valuable animal model for studying human obesity and may provide additional insights into the pathogenesis of T2DM in humans (Osto et al., 2013). Feline T2DM shares many features of human T2DM with respect to its pathophysiology, underlying risk factors and treatment strategies (Henson et al., 2006).

Mounting evidence has suggested the increasing importance of clinical recognition overweight and obesity both in dogs and cats. We hypothesize that screening changes in gene expression, related to obesity, may be valuable for early diagnosis, which in turn, allows for implementation of early interventions such as diet change, exercise, and more frequent veterinary check-ups. Gene expression analysis affords us the opportunity to study genetic contribution and patterns of altered gene expression related to obesity and T2DM. However, tissue sampling is a limitation, in particular with companion animal studies, since it is difficult to obtain permission from pet owners to use tissue samples, such as liver, adipose, and muscle for genetic studies. As such, peripheral blood can be a convenient source of cells. In particular, peripheral blood leukocytes (PBL) are increasingly considered for gene expression studies because they can be easily and repeatedly collected in sufficient quantities compared with the more invasive sampling of adipose, skeletal muscle and liver tissues. As such, PBL have been purported to be a convenient, easy to access, and readily available source of cells for sampling, with multiple studies reporting that PBL can potentially provide early warning of obesity related disorders in cats and dogs

(Kollias et al., 2011; de Mello et al., 2008; Lee et al., 2011; Lee et al., 2013).

The molecular mechanism of insulin action is directed though a complex signaling network (Cheatham et al., 1995). Insulin binding to the extracellular α-subunit of its receptor results in autophosphorylation of tyrosine residues in the receptor β-subunit and activation of a tyrosine residue intrinsic to the β-subunit (Cheatham et al., 1995). This leads to recruitment and tyrosine phosphorylation of intracellular substrates such as insulin receptor substrates (IRS) 1, 2, 3 and 4. Phosphotyrosines on the IRS proteins bind the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-K), which is a heterodimer of a regulatory subunit (p85) and a catalytic subunit (p110). Its activation, in response to insulin, results primarily through its association with the IRS proteins. Subsequently, PI3-K activation is required for GLUT4 translocation and subsequent glucose uptake. In particular, IRS-1, IRS-2 and PI3-K P-85α are important downstream players of insulin (White et al., 1998) and have been implicated with the incidence of insulin resistance and diabetes (Kerouz et al., 1997； Rondinone et al., 1997).

Recently, adiponectin has been shown to directly or indirectly affect insulin sensitivity through modulation of insulin signaling and the molecules involved in glucose and lipid metabolism (Kershaw et al., 2004). Adiponectin acts as an insulin sensitizer (Berg et al., 2001) and has a number of biological effects (Jeong et al., 2011). However, the intracellular adiponectin signaling pathway is still largely unknown in dogs and cats, despite the identification of two ADIPORs, ADIPOR1 and ADIPOR2. And some studies suggest ADIPOR1 appears to be more closely

associated with insulin secretion and sensitivity (Staiger et al., 2004). Both receptors activate signaling molecules such as AMPK (5'-AMP-activated protein kinase), PPAR-α (peroxisome proliferator-activated receptor-α) and p38 MAPK (mitogen-activated protein kinase) in vitro (Yamauchi et al., 2003).

G6PDH is a cytosolic enzyme in the pentose phosphate pathway, which supplies reducing energy to cells, by maintaining the level of NADPH. As such, G6PDH overexpression has been implicated in insulin resistance, hyperlipidemia, and increased oxidative stress in animals（Park et al., 2005: Wang et al., 2012）. MDH is an enzyme involved in gluconeogeneis, with a key role to play in the citric acid cycle. MDH reversibly catalyzes the oxidation of malate to oxaloacetate using the reduction of $NAD^+$ to NADH. Of greater quantitative importance is the production of NADPH for tissues actively engaged in biosynthesis of fatty acids and/or isoprenoids, such as the liver, mammary glands, adipose tissue and adrenal glands.

The present thesis consists of five chapters. In Chapter I, we showed that supplementing 5-point BCS with BF %, as calculated using morphometric measurements, can increase the ability for detecting overweight status in dogs, by increasing the sensitivity for detecting alterations in plasma metabolite values, induced as a result of increasing adiposity in overweight dogs. In Chapter II we compare metabolic parameters and lipid profiles between dogs with mild hyperlipidemia, and severe hyperlipidemia in order to examine the usefulness of malondialdehyde (MDA) and lipid profiles as diagnostic parameters at early stages of hyperlipidemia. In Chapter III, PBL gene expression, especially those related to

insulin and adiponectin signaling genes were carried out on lean Miniature Dachshunds and compared against similar profiles of age and gender matched overweight Miniature Dachshunds in an attempt to identify possible PBL biomarkers for assessing obesity in dogs. However, there is a lack of studies examining and determining the concordance of gene expression trends in PBL with those found in other tissues in animals. Therefore, in Chapter IV, obesity related gene transciptomes were compared and contrasted between various insulin sensitive tissues (liver, skeletal muscle, subcutaneous fat, visceral fat and peripheral blood leukocytes) from cats fed on a high fat diet. Since most obese cats studies have been based on short-term experimentally dietary manipulation, cats with long-term naturally occurring obesity have not been described adequately. Lastly, in Chapter V, the final chapter, we determined the sensitivity of PBL gene expression to short-term diet induced obesity versus long-term naturally occurring obesity in cats. We sought to observe whether different PBL transciptome patterns would emerge for obesity related genes due to different types of obesity. We believe that long-term naturally occurring obese cats are markedly more overweight, and more representative of the true clinical picture of obesity in cats and humans. As such, more experimental work needs to be done using naturally obese cats to have better representation of the clinical scenario which might lead to better early diagnosis of obesity and prevention a under clinical setting.

# Chapter I

# Using body fat percentage assessing overweight status in dogs

# Introduction

Correct estimation of body composition in dogs is important in veterinary practice and it is a tool, with which, the veterinarian can properly assess overweight status and provide owners with proper advice on feeding and weight management strategies. Body weight does not take differences in body composition into account, whereas it is reasonably well estimated using a body condition scoring (BCS) system (German et al., 2006; Mawby et al., 2004). BCS is an established inexpensive and noninvasive technique of assessing body fat percentage (BF%), commonly used in veterinary practices (Ricci et al., 2012), and a number of BCS charts are increasingly available and provide a simplified index (typically a 5- or 9-point scale) of the amount of muscle and degree of fatness of a particular animal. However, dogs deposit significant amounts of fat subcutaneously, in thoracic, lumbar and coccygeal areas as well as intra-abdominally (Burkholder et al., 2000), making the typical palpation technique associated with both BCS systems less accurate. In addition, BCS assignment is a subjective method, by nature, and although scoring systems using defined criteria can attempt to objectify the process, they cannot completely eliminate all subjectivity involved in assigning a score to an animal.

Currently in Japan, 5-point BCS is the dominant system, in spite of the fact that 9-point BCS correlates well ($r^2=0.92$) with more objective methods for evaluating BF %, such as dual-energy X-ray absorptiometry (DEXA), in both cats (Bjornvad et al., 2011) and dogs (Mawby et al., 2004). Although some have argued that using 5-point BCS, with half points, corresponds to a BCS score with 9 points,

many veterinary practitioners in Japan do not use half marks, preferring to round off and score with whole numbers instead. In addition, although BCS systems have been evaluated and validated for dogs, in most studies, the focus has been with medium to large breed dogs. Small to medium breed dogs are the preferred choice of dogs in Japan, and as such, BCS alone, may not suffice to assess overweight status. Therefore, borderline and moderately overweight dogs might not be diagnosed as being overweight and would be at risk of having no preventative care or intervention into adiposity and weight management.

As such, we sought to determine whether BF%, calculated using morphometric measurements, can increase the sensitivity for assessing overweight small-medium sized breed animals, when used in conjunction with 5-point BCS, as validated by plasma metabolite testing. Overweight or obese status in dogs, is commonly validated by plasma metabolite testing, especially with significant changes to plasma metabolites, commonly associated with lipid metabolism (Chikamune et al., 1995), therefore values for TG, T-Cho, and NEFA were focused on. In addition, BF % calculated, using morphometric measurements, has been shown to render results in agreement with DEXA value, when dogs with different genetic background and morphologic characteristics are used (Jeusette et al., 2010).

# Materials and Methods

## Animals

Twenty-five dogs which visited three different veterinary clinics in Tokyo, from June to August 2011, were prospective subjects in this study. All dogs were tested for concurrent diseases by CBC and serum biochemistry tests. In addition, case histories of all animals were examined in order to confirm their health status. Three dogs were excluded due to having disorders such as diabetes mellitus (2 dogs) and exhibiting remarkably high concentrations of plasma triglyceride and total cholesterol (1 dog), leaving 22 dogs available. Table 1 presents detailed information such as age, gender, breed, BCS and BF % of dogs.

## Subjective and Objective Body Measurements

Three veterinarians in three clinics assigned a BCS, judging by visualinspection and palpation of the amount of fat covering the rib area using a previously described five-point BCS scale (Burkholder et al., 2000). The 5 point scoring system ranges from: (1) very thin, (2) underweight, (3) ideal, (4) overweight, and (5) obese.

Body fat was calculated according to the following formulas (Mawby et al., 2004; Burkholder et al., 2000).

Male body Fat (%) = -1.4 (HS) + 0.77 (PC) + 4

Female body Fat (%) = -1.7 (HS) + 0.93 (PC) + 5

Using a simple tapemeasure, anthropometric measurements, in cm, were calculated as

previously described (Burkholder et al., 2000; Mawby et al., 2004) at the following sites: PC (pelvic circumference) and HS (Hock to Stifle). When performing measurements, a dog should stand squarely, looking straight forward with its head in a normal carriage position. The tape measure was stretched and pulled tight, until the dog's coat was just compressed against its skin. Circumferences were measured without excessive pressure or slack on the tape.

**Blood sampling**

Blood samples (≥ 4 hr postprandial) (3-4ml) were collected from the cephalic vein, without the sedation, into heparinized plastic tubes and immediately centrifuged at 1200 g for 10 min at 4$^{o}$C to obtain plasma. Samples were immediately stored at –80$^{o}$C until further use.

**Plasma metabolite, hormone concentrations, and enzyme activities assay**

Alkaline phosphatase (ALP ), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) activities, blood urea nitrogen (BUN), creatinine (CRE), glucose (GLU), total cholesterol (T-Cho), total protein (TP) and triglyceride (TG) concentrations were measured using an Olympus AU680 auto analyzer (Monolis Corporation, Tokyo, Japan) with the manufacturer's reagents. Adiponectin, nonesterified fatty acids (NEFA), insulin, were determined with the following commercial kits respectively: Dog Adiponectin ELISA Kit (CircuLex Co., Ltd. Nagano), NEFA-C test Wako (Wako Pure Chemical Industries, Inc., Tokyo), Lbis

Dog Insulin kit (SHIBAYAGI Co., Gunma).

**Statistical analysis**

Plasma metabolite data are presented as median with min/max range. Lipid metabolite values, between lean and overweight animals, were presented as vertical box plots indicating the mean, median, 10th, 25th, 75th, and 90th percentiles. Statistical significance was determined by Mann–Whitney U-test when comparing differences between two groups. The significance level was set at $p < 0.05$. Correlation strength was determined using Pearson Product Moment (r) with $p<0.05$. All tests were performed using SigmaPlot (Version. 11.2, Build 11.2.0.5, Systat Software Inc.,SanDiego,CA).

## Results and Discussion

Assessing BF % using morphometric measurements and a gender specific formula is a quick and inexpensive way to estimate BF% in a dog. However, because the absolute results of body composition analysis differ depending on the methodology used (Jeusette et al., 2010), it was imperative for us to define a working range for our study using morphometric measurements. We set our working normal reference ranges for BF% to be 15-22% for male (neutered/non-neutered) dogs, and 15-25% for female (non-spayed/spayed) dogs. BF% exceeding 22% in males, and 25% for females would be considered overweight (Table 2). Our working ranges were determined taking the following information into account. In humans, ideal BF% is 12-20% for men and 20-30% for women (Bray et al., 1993), suggesting that gender is a factor which needs to be considered. Humans are considered to be obese when BF% exceeds 20-30% of total weight (Burton et al., 1985). Alternately, dogs having approximately 15-20% body fat, not factoring gender into account, have been previously judged to be in optimal body condition (Burkholder et al., 2000). Moreover, the Kao Corporation reported the influence of gender on BF%, while measuring BF% of 5401 dogs, using theIBF-D02, a bioelectric impedance analysis (BIA) device. Female (spayed/non-spayed) and neutered male dogs had approximately 4% more BF, on average, than intact male dogs (Ishida et al., 2007), which reinforces the fact that spaying/neutering can influence body condition (Mawby et al., 2004).

Using BCS or BCS+BF % as filters, animals in our study were divided into 2

groups: lean and overweight. Plasma metabolite values, especially those related to lipid metabolism, were then compared between lean and overweight groups, according to either BCS or BCS+BF % filtering (Table 3). When using BCS alone to categorize animals, out of all the plasma lipid metabolites examined, only non-esterified fatty acid (NEFA) values of the overweight group were significantly higher than lean animals (Figure 1), with a mean ± SD value of 2.86 ± 2.48 as compared to 0.93 ± 0.81 mEq/l. Alternately, NEFA, T-Cho, and TG values of the overweight group were significantly higher than lean animals, when using BCS+BF % for filtering (Figure 1). BCS+BF % categorized overweight animals demonstrated 4x greater NEFA (3.70 ± 2.56 versus 0.87 ± 0.40 mEq/l), 0.5x greater T-Cho (311.38 ± 139.87 versus 192.86 ± 43.21 mg/dl), and 3x greater TG (226.00 ± 180.78 versus 73.50 ± 47.11 mg/dl) when compared to lean animals. As such, these results demonstrate the increased sensitivity for detecting alterations to plasma lipid metabolite values in overweight animals resulting from increased adiposity, if BF% is supplemented to BCS. Our results are supported by a previous study (Stone et al., 2009), also using BF % as a filter, which detected significant differences in T-Cho and TG of obese as compared lean dogs. The authors used a KAO IBF-D02 BIA device to determine BF % in their animals, and mean ± SD BF % for lean and obese dogs were calculated to be 25.5% ± 3.0% and 39.6% ± 1.9%, respectively. These ranges were comparable to our animals also.

The increase in sensitivity of BCS+BF %, as opposed to 5-point BCS alone, to gauge alterations to lipid metabolite values may be due to increased correlation

strength. Significant positive correlations were observed with either BCS or BF %; however, correlation strength appeared to be greater with BF %, as opposed to BCS, with glucose and lipid metabolites (Table 4). For example, BF% positively correlated with insulin ($r=0.627$, $p=0.002$; as opposed to $r=0.567$, $p=0.006$ for BCS), NEFA ($r=0.674$, $p<0.001$; as opposed to 0.614, $p=0.002$ for BCS), T-Cho ($r=0.825$, $p<0.0001$; as opposed to $r=0.643$, p=0013 for BCS), and TG ($r=0.5823$, $p<0.005$; as opposed to $r=0.533$, $p=0.011$ for BCS). In addition, BF % exhibited correlations with liver status metabolites by correlating positively with BUN ($r=0.429$, $p<0.05$), CRE ($r=0.490$, $p=0.021$), and TP ($r=0.737$, $p<0.0001$; as opposed to 0.500, $p=0.02$ for BCS) which all tend to increase as a result of incurred liver damage from increasing adiposity. BCS correlated negatively with LDH ($r=-0.470$, $p=0.03$), a marker for energy metabolism, which has been shown to decrease with increasing adiposity (Nadler et al., 2000). Overall, since 5-point BCS and BF % exhibit different correlation strengths with different plasma metabolites, both parameters complement one another and should be used in tandem, in order to better assess overweight status in dogs.

Interestingly, adiponectin levels did not significantly differ between lean and overweight animals categorized by either BCS or BCS+BF %. The relationship between adiposity and plasma adiponectin levels in dogs has yet to be unequivocally demonstrated with some studies indicating an inverse relationship (Ishioka et al., 2006; Mori et al., 2012; Mori et al., 2011), no relationship (German et al., 2009; Wakshlag et al., 2010; Verkest et al., 2011), and even a positive relationship (Mori et al., 2010; Grant et al., 2011) Four possible factors which may confound the

relationship between adiposity and adiponectin concentration in dogs are neuter status（Verkest & Rose et al., 2011）, type of obesity（Verkest & Rand, 2011）, breed and gender (Mori et al., 2010; Grant et al., 2011) and diet（Pischon et al., 2005; Silva et al., 2011. Our BCS filtered overweight group consisted of 11 animals, 5 males (4 neutered/1 intact) and 6 females (3 spayed/ 3 intact); whereas our BF % filtered overweight group consisted of 8 animals, 4 males (3 neutered/ 1 intact) and 4 females (1 spayed/ 3 intact). The increased polarity of a female hormonal profile of the animals in the BF % filtered, as compared to the BCS filtered group may explain the trend of increasing adiponectin observed (Mori et al., 2010; Grant et al., 2011). Hence, details concerning the three aforementioned factors may be required on any future adiposity studies in dogs involving adiponectin, in order to better explain their results, since the relationship between adiposity and adiponectin in dogs is not clear as of yet. As it stands currently, plasma adiponectin levels cannot be reliably used as a gauge for adiposity in dogs.

This study has a number of limitations. First, BF% can be estimated clinically based on simple gender specific calculations using morphometric measurements, however accuracy is reduced due to the variety of body proportions in different dog breeds. Since breed dependence is a factor (Jeusette et al., 2010), tailoring the equation to the actual breed may be necessary to gain better accuracy for determining body composition when using morphometric measurements alone to calculate BF %. Further clinical studies are required to compare BF% calculated from morphometric measurements among different dog breeds, to determine breed specific compensatory

factors for the formulas. Second, non-fasted blood samples were obtained from client-owned dogs in this study.

It is impossible to eliminate any postprandial effect completely even if the samples were collected at least 4 hrs after feeding, especially on plasma metabolites values such as NEFA, T-Cho, and TG. However, since no significant differences in plasma insulin or glucose values were observed amongst the dogs, any postprandial effect present may be minimal. In addition, using non-fasted blood samples, may arguably be more representative of a true clinical setting. Third, age was not factored or considered as an influence factor in our criteria for categorizing obesity, although it has been shown to be known risk factor for the development of obesity (Lund et al., 2006). Fourth, the sample size of the groups was small. A larger sample size of animals should be used in a future study to determine reproducibility of our results and to increase statistical power of the results. Lastly, the accuracy of 5-point BCS or BF% (using morphometric measurements) to assess overweight status cannot be evaluated and compared due to a lack of comparison with a gold-standard method of measuring fat mass, such as DEXA or MRI or isotope dilution. We hope to have access to a Kao Corporation (Tochigi, Japan) IBF-D02 bioelectric impedance device, which has been previously described to objectively determine and measure BF% in future studies.

# Conclusion

In conclusion, our data indicates that supplementing 5-point BCS with BF %, as calculated using morphometric measurements, can increase the ability for detecting overweight status in small-medium sized breed dogs, by increasing the sensitivity for detecting alterations in plasma metabolite values, especially lipid metabolites such as NEFA, T-Cho, and TG, induced as a result of increasing adiposity in overweight dogs. Therefore, BF % should complement the 5-point BCS system and be used in tandem, in order to better detect and diagnose overweight status in dogs, allowing for possible early intervention and prevention of development of more advanced stages of obesity.

# Tables and Figures

Table 1. Physical characteristics of the dogs used in this study

| Breed | Spaying/Neutering Status | Gender | Age (years) | Body Weight (Kg) | Body Condition Score (BCS out of 5) | Anthropometric Measurements | | Body Fat % (BF %) |
|---|---|---|---|---|---|---|---|---|
| | | | | | | PC (cm) | HS (cm) | |
| Beagle | - | Female | 9 | 11.9 | 2.5 | 42 | 19 | 11.76 |
| Labrador | - | Male | 2 | 31.0 | 3 | 57 | 22 | 17.09 |
| Chihuahua | - | Female | 2 | 2.7 | 3 | 27 | 5.5 | 19.37 |
| Shih Tzu | + | Male | 3 | 5.4 | 3 | 29.5 | 6 | 18.32 |
| Beagle | - | Female | 5 | 13.1 | 3 | 45.5 | 19 | 15.02 |
| Miniature Dachshund | - | Female | 5 | 5.0 | 3 | 31.5 | 4.5 | 26.65 |
| Beagle | - | Female | 7 | 13.8 | 3 | 49.5 | 19 | 18.74 |
| Miniature Dachshund | - | Male | 7 | 4.7 | 3 | 31.5 | 6.5 | 19.16 |
| Beagle | - | Female | 9 | 12.1 | 3 | 46 | 19 | 15.48 |
| Beagle | + | Female | 7 | 14.5 | 3.5 | 52 | 17.5 | 23.61 |
| Beagle | + | Female | 7 | 14.5 | 3.5 | 48 | 18 | 19.04 |
| Miniature Dachshund | - | Female | 6 | 5.0 | 4 | 31 | 6.5 | 22.78 |
| Long foot Chihuahua | + | Male | 7 | 6.3 | 4 | 36 | 9 | 19.12 |
| Chihuahua | + | Female | 8 | 2.7 | 4 | 25.5 | 5.5 | 19.37 |
| Yorkshire Terrier | + | Male | 9 | 4.2 | 4 | 40 | 7 | 25.00 |
| Beagle | - | Female | 9 | 14.7 | 4 | 55 | 18 | 25.55 |
| Shih Tzu | + | Female | 13 | 7.6 | 4 | 37 | 9.5 | 23.26 |
| Shih Tzu | - | Male | 14 | 6.3 | 4 | 39 | 6 | 25.63 |
| Miniature Dachshund | - | Male | 5 | 8.4 | 4.5 | 45.6 | 12 | 22.31 |
| Chihuahua | + | Female | 7 | 5.65 | 4.5 | 43 | 11 | 26.29 |
| Mix | - | Female | 10 | 20 | 5 | 71 | 14.5 | 46.38 |
| Beagle | + | Male | 15 | 15 | 5 | 65 | 12 | 37.25 |

Table 2. Body fat reference ranges by morphometric measurements

| | Normal | Overweight |
|---|---|---|
| Female (Intact/Neutered) | 15-25% | >25% |
| Male (Intact/Neutered) | 15-22% | >22% |

Table 3
Clinical characteristics and plasma metabolite concentrations

| | Clinical Parameters | Determined by BCS | | Determined by BCS+BF% | |
|---|---|---|---|---|---|
| | | Lean (n=11) | Overweight (n=11) | Lean (n=14) | Overweight (n=8) |
| Physical Indexes | Age (years) | 7.0 (2.0-9.0) | 9.0* (5.0-15.0) | 7.0 (2.0-13.0) | 9.0 (5.0-15.0) |
| | Body Condition Score (1-5) | 3.0 (2.5-3.5) | 4.0* (4.0-5.0) | 3.0 (2.5-4.0) | 4.3* (3.0-5.0) |
| | Amount Body Fat (%) | 18.74 (11.76-26.65) | 25.0* (15.94-46.38) | 19.08 (11.76-23.61) | 25.96* (22.31-46.38) |
| | Pelvic Circumference (cm) | 45.5 (27.0-57.0) | 40.0 (25.5-71.0) | 39.5 (25.5-57.0) | 44.3 (31.5-71.0) |
| | Hock to Stifle Joint Length (cm) | 18.0 (4.5-22.0) | 9.5 (5.5-18.0) | 13.5 (5.5-22.0) | 11.5 (4.5-18.0) |
| DM | Glucose (mg/dl) | 95.0 (80.0-111.0) | 97.0 (78.0-116.0) | 98.0 (80.0-116.0) | 92.0 (78.0-110.0) |
| | Insulin (ng/ml) | 1.05 (0.16-2.33) | 1.59 (0.32-5.44) | 1.23 (0.16-5.15) | 1.53 (0.32-5.44) |
| Obesity | Adiponectin (mg/ml) | 24.00 (9.04-65.09) | 16.09 (6.23-49.86) | 18.76 (7.40-65.09) | 28.57 (6.23-49.39) |
| | Non-estrified fatty acids (mEq/l) | 0.73 (0.23-3.14) | 1.50* (0.78-9.01) | 1.01 (0.23-1.50) | 3.39* (0.78-9.01) |
| | Total Cholesterol (mg/dl) | 191.0 (133.0-290.0) | 245.0 (145.0-594.0) | 188.00 (133.0-290.0) | 314.50* (149.00-594.00) |
| | Triglycerides (mg/dl) | 67.0 (31.0-215.0) | 105.0 (19.0-645.0) | 67.0 (19.0-215.0) | 184.0* (87.0-645.0) |
| Hepatic and Renal Injury | Alanine aminotransferase (U/l) | 45.0 (32.0-186.0) | 56.0 (29.0-214.0) | 50.5 (32.0-186.0) | 64.0 (29.0-214.00) |
| | Alkaline Phosphatase (U/l) | 211.0 (83.0-373.0) | 161.5 (68.0-871.0) | 216.0 (83.0-788.0) | 111.0 (68.0-871.0) |
| | Aspartate aminotransferase (U/l) | 27.2 (16.4-74.9) | 25.9 (18.6-32.5) | 27.2 (16.4-74.9) | 25.8 (18.6-32.5) |
| | Blood Urea Nitrogen (mg/dl) | 15.1 (14.0-23.5) | 15.4 (11.9-32.7) | 15.1 (11.9-32.7) | 17.8 (13.4-27.7) |
| | Creatinine (mg/dl) | 0.82 (0.56-1.01) | 0.69 (0.49-1.24) | 0.75 (0.49-1.01) | 0.76 (0.69-1.24) |
| | Lactate dehydrogenase (U/l) | 185.0 (69.0-404.4) | 106* (34.0-247.0) | 145.5 (69.0-404.0) | 109.0 (34.0-247.0) |
| | Total Protein (g/dl) | 6.5 (6.1-7.1) | 6.8 (5.9-7.7) | 6.5 (6.1-7.1) | 7.0 (5.9-7.7) |

DM=Diabetes Mellitus
Values are presented as median with (range)
* denotes significance when compared against corresponding healthy group ($p<0.05$, Mann-Whitney U-test)

Table 4
Correlation between BCS or BF% with plasma metabolite values

| Plasma Metabolites | BCS ($r$) | $p$ value | BF% ($r$) | $p$ value |
|---|---|---|---|---|
| Glucose | -0.001 | 0.997 | -0.175 | 0.437 |
| Insulin | 0.567 | 0.0059 | 0.627 | 0.0019 |
| Adiponectin | -0.098 | 0.665 | 0.187 | 0.405 |
| NEFA | 0.614 | 0.0024 | 0.674 | 0.0006 |
| Cholesterol | 0.643 | 0.0013 | 0.825 | <0.0001 |
| Triglycerides | 0.533 | 0.0107 | 0.583 | 0.0044 |
| ALT | 0.266 | 0.231 | 0.301 | 0.173 |
| ALP | 0.059 | 0.803 | -0.016 | 0.945 |
| AST | -0.274 | 0.217 | -0.249 | 0.265 |
| BUN | 0.386 | 0.0756 | 0.429 | 0.0462 |
| CREA | 0.173 | 0.442 | 0.490 | 0.0207 |
| LDH | -0.470 | 0.0271 | -0.370 | 0.0905 |
| TP | 0.500 | 0.0177 | 0.737 | <0.0001 |

Pearson Product Moment Correlation Coefficient ($r$) is expressed to determine correlation strength. Shaded areas indicate a significant strength of correlation with $p$ <0.05.

Figure 1

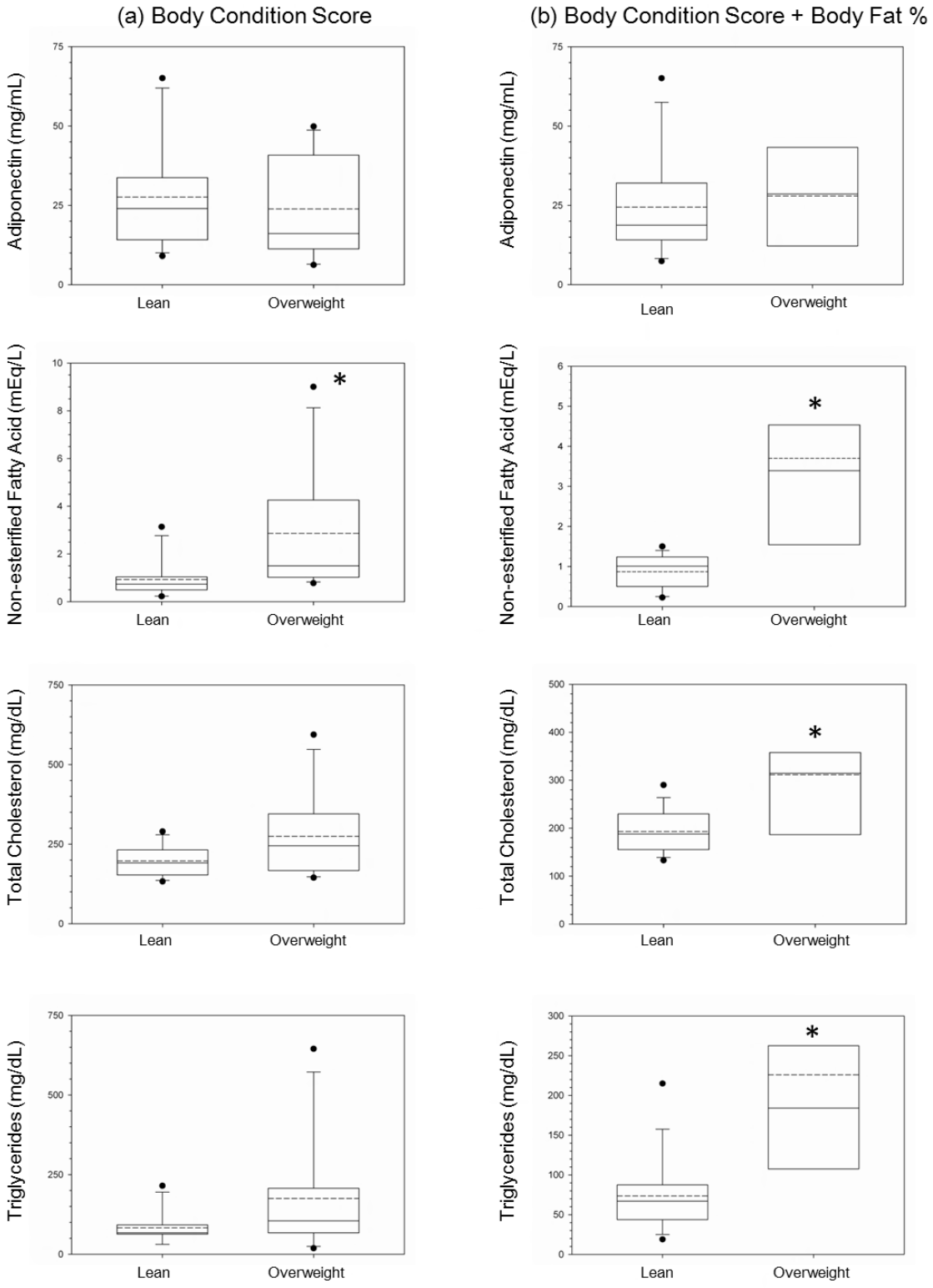

(a) Body Condition Score
(b) Body Condition Score + Body Fat %
Adiponectin (mg/mL)
Non-esterified Fatty Acid (mEq/L)
Total Cholesterol (mg/dL)
Triglycerides (mg/dL)
Lean
Overweight
*

**Figure 1**

Plasma lipid metabolite comparison between lean and overweight animals as filtered using body condition score (BCS-Panel a) or body fat percentage (BCS+BF%-Panel b). Data are shown as vertical box plots indicating the $10^{th}$, $25^{th}$, $75^{th}$, and $90^{th}$ percentiles as vertical boxes with error bars. Median and mean are indicated by solid and dashed horizontal lines, respectively whereas outliers are represented as dots. Asterisk (*) indicates significantly higher when compared against lean group (Mann-Whitney U-Test, $p<0.05$).

# Chapter II

# Plasma lipoprotein profiles and malondialdehyde in hyperlipidemia dogs

# Introduction

Aberrations in plasma cholesterol and triglyceride levels are indicative of diseases associated with obesity and diabetes mellitus (Bauer et al., 2004). Mounting evidence has suggested the increasing recognition of clinical importance of hyperlipidemia in dogs (Fleeman et al., 2009; Xenoulis et al., 2010). However, many of hyperlipidemia dogs physically appear healthy and do not usually exhibit any symptoms. Therefore, many veterinary practitioners and dog owners exhibit little interest in mild lipid metabolism abnormality. Only when the disease progresses and the dog become severely hyperlipidemic, they initiate therapy with anti-hyperlipidemic drugs. In 2012, we set the new screening criteria for detecting early stages of hyperlipidemia in dogs (Kawasumi et al., 2012). When we evaluated apparently healthy dogs using these criteria, 23.7 % dogs (9 of healthy 38 dogs) were diagnosed as mild hyperlipidemia. We think that the use of hyperlipidemia screening is valuable in early diagnosis, which in turn, allows for an implementation of early interventions such as diet change, exercise, and more frequent veterinary check-ups. In humans, plasma lipid profile analysis can be useful in diagnosing lipid metabolic disorders.

Lipoproteins are believed to play important roles in energy and lipid metabolism of animals, and can reflect metabolic changes. Alterations in the dog's lipoprotein cholesterol fractions are indicative of aberrations of lipid metabolism due to hyperlipidemia and/or obesity as in human (Mori et al., 2011). Hyperlipidemia induces oxidative stress, and malondialdehyde (MDA) is one of the end products in lipid peroxidation. Plasma MDA levels increased markedly in animals with obesity and

diabetes mellitus and increased MDA indicated elevations of lipid oxidation in tissues (Moussa et al., 2008). Humans with apparent increase in Malondialdehyde-modified LDL were shown to be more predisposed to developing arteriosclerosis. However it should be noted, dog's lipoprotein density profiles and lipid metabolism are different from those of humans (Terpsta et al., 1982; Mori et al., 2011).

The aim of this chapter is to compare metabolic parameters, MDA as a lipid oxidation marker, and lipid profiles between dogs with mild hyperlipidemia, and severe hyperlipidemia in order to examine the usefulness of MDA and lipid profiles as diagnostic parameters at early stages of hyperlipidemia.

# Materials and Methods

## Animals

Twenty one dog samples were collected from four different veterinary clinics across Japan (Ibaraki prefecture and Kanagawa prefecture) from March to June 2013. Thirteen dogs (age: median 8.0 years old; number of females (F): 8; number of males (M): 5; body weight: median 5.6kg) were classified as healthy control subjects by each clinical veterinarian. 4 dogs (age: median 12.5 years; M: 4; body weight: median 8.9 kg) under anti-hyperlipidemic therapy (statins and fibrates) were diagnosed as severely hyperlipidemic. The remaining 4 dogs (age: median 9.5 years; F: 3; M: 1; body weight: median 7.7 kg) were diagnosed as mildly hyperlipidemic based on the screening of hyperlipidemia as follow (Kawasumi et al., 2012). A subject may be classified as mildly hyperlipidemic if the biochemical analysis of the plasma shows any two of the following 3 factors:

1) Elevated triglyceride level >165mg/dL

2) Elevated total cholesterol level >200mg/dL

3) Elevated non-esterified fatty acids level >1.5mEq/ L

## Plasma metabolite and hormone concentrations and enzyme activities assay

Postprandial blood samples (at least 4 hours after the last meal) were collected from the forelimb vein into heparinized plastic tubes, and plasma was recovered via centrifugation at 4°C and stored at –25°C until further use. Plasma glucose, triglycerides(TG), total cholesterol, total protein, aspartate aminotransferase, and

alanine aminotransferase(ALT), alkaline phosphatase(ALP), albumin, albumin verses globulin ratio, total bilirubin, total bile acids, γ-GTP were measured by auto-analyzer (Monolis, Inc ,Tokyo, Japan) using the manufacturer's reagents. Nonesterified fatty acids, adiponectin, insulin and MDA were determined with NEFA-C test Wako (Wako Pure Chemical Industries, Inc., Tokyo), Dog adiponectin ELISA kit (Circulex Co., Ltd, Nagano), and Lbis dog Insulin kit (Shibayagi Co., Gunma), Malondialdehyde Assay kit, (Northwest Life Science Specialties LLC, Vancouver, WA) respectively.

**Cholesterol lipoprotein profiling**

In order to examine lipoprotein fractions of cholesterol and triglyceride for all dogs, we performed biphasic agarose gel electrophoresis method using commercial Quickgel Lipo gels (Helena Laboratories, Saitama). Lipoproteins were separated by electrophoretic technique and analyzed using a Helena Laboratories Epalyzer 2 Electrophoresis processing Analyzer. Lipoprotein fractions were assessed and analyzed using Edbank III analysis software (Helena Laboratories, Saitama, Japan). Lipoprotein electrophoresis pattern consisted of 4 bands. The bands from left to right were: α1-migrating lipoproteins ($HDL_2$ and $HDL_3$), α2-migrating lipoproteins ($HDL_1$), β-migrating lipoproteins (LDL and VLDL), and κ band (small % of chylomicrons) remaining at the origin (Mori et al., 2011).

**Statistical analysis**

Values were expressed in medians with inter-quartile range (25th Percentile and 75th Percentile). Statistical significance was determined by One-way Analysis of Variance by Ranks among groups. All tests were performed using SigmaPlot version.11.2 (Systat Software Inc., San Diego, CA, USA). The significance level was set at $P < 0.05$.

# Results

As shown in Table 1, triglycerides level of untreated hyperlipidemia group was significantly higher than those of control group. ALT level of hyperlipidemia with treatment group was the highest among three groups. ALP level of hyperlipidemia dog with treatment was significantly higher than those of control and untreated hyperlipidemia dogs. Although no significant difference in total cholesterol concentration was observed, total cholesterol level of hyperlipidemic dogs with treatment tended to be higher compared to the other groups. VLDL and LDL in cholesterol and triglyceride of untreated hyperlipidemia dogs were the highest among three groups. HDL1 level in triglyceride of hyperlipidemia with treatment group was significantly higher than those of control and untreated hyperlipidemic groups. Chylomicron level in triglyceride of untreated hyperlipidemia dogs was higher than those of control and hyperlipidemic dogs with treatment.

Figure 1 illustrates the comparison of MDA concentrations among control, untreated hyperlipidemia and hyperlipidemia with treatment dogs. MDA concentration of untreated hyperlipidemia dogs was significantly higher than those of control and hyperlipidemic dogs with treatment.

## Discussion

Various diagnostic criteria for hyperlipidemia have already been established. The first states plasma total cholesterol level of over 300mg/dl as a criterion (Whitney et al., 1992), and another sets both plasma triglyceride and plasma cholesterol levels to be over 500mg/dl (Xenoulis et al., 2010). The above methods only detect dogs which are in severely hyperlipidemic condition and in need of clinical interventions. Our screening methods aim to detect early stages of hyperlipidemia in dogs that may be missed by veterinary practitioners. In current study, abnormal lipid status in plasma was clearly detected in mild hyperlipidemia group. In this study, all of the severely hyperlipidemic dogs were under anti-hyperlipidemic therapy as recommended by their veterinarians. The therapy appeared to be fully effective since triglyceride level in hyperlipidemia with treatment group was lower than those of untreated hyperlipidemia group and there was no significant difference in plasma cholesterol levels among three groups. At the time of the blood draw, three of four severely hyperlipidemic dogs were on fibrates and one dog was on statins. The fibrates are considered the most effective hypertriglyceridemia drugs (Brunzell et al., 1982) and the statins are a class of LDL-cholesterol lowering drugs (Bilheimer et al., 1983; Tamura et al., 2003). ALT levels in hyperlipidemic dogs with treatment were expected to be elevated due to hepatic metabolism of these drugs. HDL1 is a large floating particle which plays an important role in reverse cholesterol transport. LDL functions similarly as $HDL_1$ and is unique to dogs (Mori et. al., 2011). HDL1 fractions of both cholesterol and

triglycerides in hyperlipidemia with treatment group were the highest among three groups.

Anti-hyperlipidemic drugs were also effective in reducing lipid oxidation. MDA concentration in untreated hyperlipidemic dogs was the highest among three groups. MDA is an end product of lipid peroxidation, and MDA-modified LDL (MDA-LDL) is generated via lipid peroxidation or during platelet aggregation. Both statins and fibrates therapies had a positive effect on reducing serum MDA-LDL concentrations in human lipid metabolic mechanism (Kondo et al., 2002; Kanno et al., 2004). Also statins and fibrates in dogs are effective in relieving the increased lipids and lipid oxidation. Untreated hyperlipidemia group showed significantly higher plasma triglycerides concentration than control and hyperlipidemia with treatment groups. Associated VLDL and LDL in lipoprotein fractions were also higher than those of control and hyperlipidemia with treatment groups. As untreated hyperlipidemia dogs showed abnormality in lipid metabolism, our new criteria in hyperlipidemia is shown to be beneficial in metabolic disease prevention. Xenoulis et al. (2013) also reported in current paper that lipoprotein profiles in dogs could potentially be useful as diagnostic tools in identification of dogs suspected of having lipoprotein abnormalities.

# Conclusion

In this study, dogs with untreated hyperlipidemia clearly showed abnormal lipid status, whereas hyperlipidemic dogs under anti-hyperlipidemia treatment showed more normal lipid status suggesting the effectiveness of the therapy. Plasma lipid (triglycerides and cholesterol) profiles and MDA may be useful as diagnostic tools for early stages of hyperlipidemia dogs.

# Tables and Figures

Table 1. Comparison of plasma baiomarker levels and lipoprotein profiles among control, hyperlipidemia dogs with/without treatment.

| Group(n) | Control (13) | Hyperlipidemia | |
|---|---|---|---|
| | | Untreatment (4) | With treatment (4) |
| Age | 8.0 (2.8-9.3) | 9.5 (6.0-11.5) | 12.5 (12.0-15.0) |
| Body condition score | 3.0 (3.0-4.0) | 3.5 (2.3-4.8) | 3.0 (3.0-4.0) |
| Body weight (kg) | 5.6 (4.3-14.5) | 7.7 (3.0-21.3) | 8.9 (5.6-26.9) |
| Total choresterol(mg/dL) | 240 (170-351) | 241 (231-358) | 367 (347-390) |
| Triglycerid (mg/dL) | 48 (28-163) | 252 (196-311)* | 81 (61-88) |
| Total Protein (g/dL) | 6.8 (6.4-7.4) | 7.5 (6.7-8.0) | 7.3 (7.1-7.7) |
| Albmin (g/dL) | 3.3 (3.0-3.4) | 3.4 (3.0-3.8) | 3.3 (3.0-3.6) |
| Albumin/Globulin ratio | 0.88 (0.79-0.97) | 0.83 (0.80-0.89) | 0.78 (0.76-0.88) |
| Total bilirubin (mg/dL) | 0.1 (0.1-0.2) | 0.2 (0.1-0.2) | 0.1 (0.1-0.2) |
| AST (IU/L) | 33 (24-39) | 35 (20-38) | 33 (26-45) |
| ALT (IU/L) | 44 (37-67) | 40 (24-65)** | 123 (75-315)* |
| ALP (IU/L) | 127 (72-218) | 335 (101-2259) | 877 (318-1113)* |
| γ-GTP (IU/L) | 6.1 (3.9-7.7) | 5.1 (2.8-7.7) | 7.7 (6.0-35.8) |
| Total bile acid (mg/dL) | 5.7 (2.5-23.5) | 15.8 (6.4-104.3) | 10.4 (6.2-31.2) |
| Glucorse (mg/dL) | 90 (81-110) | 102 (96-109) | 103 (90-375) |
| Free fatty acids (mEq/L) | 0.78 (0.64-0.97) | 0.73 (0.55-1.14) | 0.52 (0.41-0.65) |
| Insulin (ng/mL) | 1.1 (0.8-1.7) | 0.7 (0.6-1.1) | 2.4 (1.2-2.5) |
| Adiponectin (μg/mL) | 18.4 (15.9-30.1) | 31.9 (17.1-38.9) | 23.8 (19.0-25.9) |
| Cholesterol profiles | | | |
| HDL2,3(mg/dl) | 191.8 (143.1-225.0) | 191.2 (167.2-240.0) | 229.7 (215.8-242.8) |
| HDL1(mg/dl) | 31.1 (24.3-85.5) | 41.7 (25.2-124.4) | 119.9 (96.3-136.6) |
| VLDL/LDL(mg/dl) | 5.8 (3.3-14.4) | 22.5 (16.5-35.7)* | 21.5 (7.7-35.9) |
| CM(mg/dl) | 0.0 (0.0-0.2) | 1.5 (0.0-6.7) | 0.0 (0.0-0.0) |
| Triglyceride profiles | | | |
| HDL2,3(mg/dl) | 0.0 (0.0-0.0) | 0.0 (0.0-0.0) | 0.0 (0.0-0.2) |
| HDL1(mg/dl) | 0.5 (0.0-1.8) | 0.9 (0.75-5.0) | 3.0 (1.9-6.5)* |
| VLDL/LDL(mg/dl) | 47.5 (28.1-108.4) | 193.1 (119.0-264.5)*,** | 67.9 (59.7-72.3) |
| CM(mg/dl) | 0.5 (0.0-48.7) | 58.6 (0.0-119.3) | 5.6 (0.0-14.5) |

Values are presented as median.Numbers in parentheses present from 25% to 75% value.

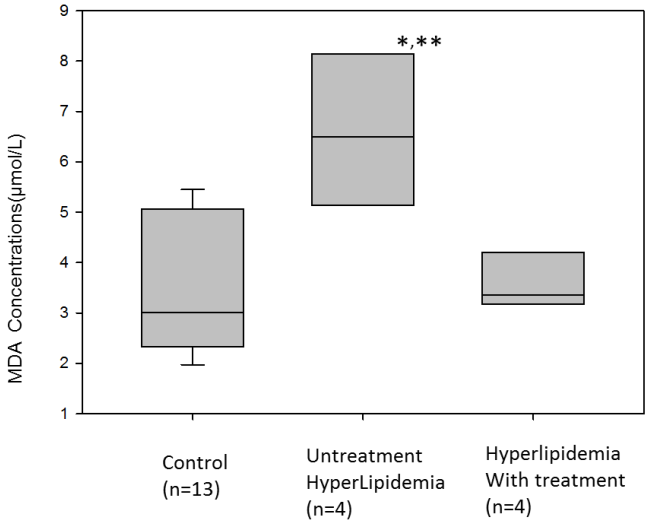
* Significantly different ($p<0.05$) from the values of Control group by Kruskal-Wallis One Way Analysis of Variance on Ranks.

** Significantly different ($p<0.05$) from the values of hyperlipidemia with treatment group by Kruskal-Wallis One Way Analysis of Variance on Ranks.

All dogs were also determined by palpation and inspection on with a body condition score (BCS: a 5 point scale) the five-point scale: (1) very thin, (2) underweight, (3) ideal, (4) overweight, and (5) obese.

Figure 1

Change in MDA concentrations among control, hyperlipidemia dogs with / without treatment.




Graph is expressed as median with inter-quartile range (25th Percentile and 75th Percentile).
* Significantly different (p<0.05) from the values of Control group by Kruskal-Wallis One Way Analysis of Variance on Ranks.
** Significantly different (p<0.05) from the values of Severe hyperlipidemia group by Kruskal-Wallis One Way Analysis of Variance on Ranks.

## Figure legend

**Figure 1**

**Change of plasmaMDA concentrations among control, mild and severe hyperlipidemia dogs.**

Graph is expressed as median with inter-quartile range (25th Percentile and 75th Percentile).

* Significantly different (p<0.05) from the values of Control group by Kruskal-Wallis One Way Analysis of Variance on Ranks.

** Significantly different (p<0.05) from the values of hyperlipidemia with treatment group by Kruskal-Wallis One Way Analysis of Variance on Ranks.

# CHAPTER III

# Insulin and adiponectin gene expression of PBL in obese Miniature Dachshunds

# Introduction

Gene expression analysis of PBL, in particular peripheral blood mononuclear cells (PBMC) has previously been used for investigating the molecular mechanisms underlying several human diseases, as surrogates for predicting potential effects in tissues, that are not easily accessible (Eady et al., 2005). Interestingly, a remarkable concordance (80%) of gene expression profiles between PBMC and different tissues has been previously demonstrated (Liew et al., 2006). Moreover, PBMC have been shown to be sensitivity to changes in gene expression of genes involved in energy homeostasis (Caimari et al., 2010a) and sterol metabolism (Caimari et al., 2010) resulting from acute changes in feeding conditions of rats. Lastly, PBMC enzymes are considered to be a useful marker to evaluate the energy metabolism condition of animals (Magori et al., 2005; Takeguchi et al., 2005).

Therefore, the aim of this chapter was to assess whether PBL are sufficiently sensitive enough for use in determining changes in gene expression of genes related to insulin (IRS-1, IRS-2, PI3-K,) and adiponectin (ADIPOR1, ADIPOR2) signaling gene pathway, energy homoeostasis (MDH, G6PDH) and lipid metabolism (FAS), at the mRNA level, by RT-PCR between age matched healthy overweight and lean Miniature Dachshund, which is one of the easiest obesity breeds (Zoran DL., 2010). If so, PBL can serve as an easily accessible cell type, for possibly detecting overweight status and subsequent obesity risk, by indirectly monitoring for alterations in gene expression of genes related to insulin and adiponectin signaling genes.

# Materials and Methods

## Animals

Nine Miniature Dachshund (two intact males, two intact females, three neutered male and two spay females; 5-9 years old), which presented themselves at private veterinary clinics in Tokyo, Japan for routine checkups between April and May 2012. None of the selected animals had any evidence of acute or chronic diseases (except for overweight/obesity) based on physical and clinical examination of routine hematologic and biochemical analysis. The body score condition (BCS) of each dog was assessed using a five point scale (Burkholder et al., 2000) by the veterinarian working in the clinic, using the amount of fat covering the rib area, as judged by visual inspection and palpation. Body fat % was measured with a commercially available Kao IBF-D02 bioelectric impedance device (Kao Corporation, Tokyo) according to the manufacturer`s instructions. The IBF-D02 has been shown to be able to objectively quantify canine BF% under a clinical setting (Stone et al., 2009).

## Blood collection

Blood samples (≥ 4 h postprandial) (4-5mL) were collected from the cephalic vein of animals without the aid of sedation. 3mL of this blood was collected into PAXgene Blood RNA V.2 kit tubes (PreAnalytiX GmbH) for RNA stabilization, preservation, and sample transport. Tubes were inverted ten times, maintained at room temperature for 2 h, frozen at -20 $^{o}$C overnight and subsequently moved to -80 $^{o}$C for

storage until further use. The remainder of blood was collected into heparinised plastic tubes, for immediate centrifugation at 1200 *g* for 10 min at 4 °C to obtain plasma which was immediately stored at -80 °C until required.

**Analysis of plasma metabolites and hepatic enzymes**

Plasma glucose, blood urea nitrogen (BUN), creatinine (CRE), total cholesterol (T-Cho), total protein (TP), and triglyceride (TG) concentrations, as well as lactate dehydrogenase (LDH), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activities, plasma nonesterified fatty acid (NEFA), adiponectin and immunoreactive insulin (IRI) concentrations were determined as previously described in Chapter I.

**Quantitative real-time PCR analysis of peripheral blood leucocyte mRNA**

Total leukocyte RNA from the blood samples was extracted and isolated using a PAX gene Blood RNA V.2 kit and a QIAamp RNA Blood Mini Kit according to the manufacturer's instructions. The concentration of RNA was assessed by e-spect malcom (No.08728036). Quantitative RT-PCR reactions, using SYBR Green fluorescent dye, for genes of interest (IRS-1, IRS-2, PI3K p85 α, MDH, G6PDH, FAS, ADIPOR1, ADIPOR2) were performed in triplicate, with 18s rRNA serving as internal standards, using an ABI 7300 RT-PCR sequence detection system. Primers for the genes are listed (Table 1).

Relative gene expression values were calculated using the comparative $C_T$

method for quantification as described by (Livak et al. 2001). All target genes were normalized to 18s rRNA and subsequently compared to ‘control’ data to determine relative *n*-fold differences.

**Statistical analysis**

Plasma metabolite values were expressed as mean ± standard deviation (SD). qRT-PCR relative expression values were expressed as vertical box plots indicating mean, median, 10th, 25th, 75th, and 90th percentiles. The Mann-Whitney *U* Test was used to assess significance between groups, set at $P < 0.05$. Analysis was performed using SigmaPlot (Version. 11.2, Build 11.2.0.5, Systat Software Inc., San Diego, CA).

# Results

## Analysis of plasma metabolites and hepatic enzymes

Working reference ranges for lean and overweight classification were determined to be 20-25% for non-neutered male dogs, and 25-30% for female (non-spayed/spayed) and neutered male dogs. BF% exceeding 25% in intact males, and 30% for females (intact or spayed) and neutered males was considered to be overweight.

Clinical characteristics and mean plasma values of general plasma metabolites and hepatic enzymes are presented in Table 2. Overweight status resulted in a significant increase ($P<0.05$, Mann-Whitney *U* Test) in plasma NEFA, T-Cho, triglycerides and ALT, and a significant decrease ($P<0.05$, Mann-Whitney *U* Test) in plasma adiponectin, as compared to lean Miniature Dachshund.

## Quantitative RT-PCR comparative analysis of peripheral blood leukocyte mRNA between lean and overweight dogs

Comparison of PBL mRNA expression trends between overweight and lean Miniature Dachshund PBL is presented in Figure 1. With regards to insulin and adiponectin signaling activity and lipid synthesis overweight Miniature Dachshund PBL demonstrated a significant ($P<0.001$, Mann-Whitney *U* test) median reduction in IRS-1, IRS-2, PI3K P85α, ADIPOR1 and FAS mRNA expressions, as compared to control dogs PBL.

## Discussion

This chapter showed that as compared to lean dogs, obesity resulted in higher circulating levels of TG (2x greater), T-Cho (50% greater), ALT (2x greater) and NFFA (2x greater), respectively. These findings confirm previous data obtained in obese dogs and other species (Bailhache et al., 2003; Staiger, et al., 2004; Hatano et al., 2010) and may provide further evidence of changing metabolic status of their weight gain. It has been shown that the elevated plasma TG and T-Cho concentrations may pose a risk for pancreatic disorders in dogs (Umeda et al., 2006). And some studies hypothesis that the change in plasma NEFA levels may be the signal for hyperinsulinemic compensation (Stefanovski et al., 2011).

Alteration to weight (increase or decrease), when associated with obesity, has been reported to lead to alterations to PBL gene expression (Ghanim et al., 2004; de Mello et al., 2008a), especially those related to insulin (de Mello et al., 2008; Mori et al., 2009) and adiponectin (Kollias et al., 2011) signaling genes. IRS-1, IRS-2 have been implicated with the incidence of insulin resistance and diabetes (Kerouz et al., 1997; Rondinone et al., 1997). Lower protein levels of IRS-1, IRS-2and PI3-K p85α have been reported in human patients suffering from insulin resistance (Rondinone et al., 1997; Friedman et al., 1999). qRT-PCR revealed a significant reduction in IRS-1, IRS-2 and PI3K p85α mRNA expression by PBL of overweight, as compared to healthy lean animals, which is supported by a previous study with cats, conducted by our laboratory (Mori et al., 2009). Coincidentally, increased circulating insulin concentrations were also observed in overweight as compared to healthy lean dogs,

which would corroborate with the reduction of insulin signaling gene mRNA expression observed. It has previously been shown that dogs with chronic, naturally occurring obesity compensate for obesity-induced insulin resistance by secreting more insulin after a glucose challenge (Bergman et al., 2001).

Adiponectin plays a significant role in obesity, insulin resistance, and T2DM in humans, acting through two cell membrane receptors, ADIPOR1 and R2. Little is known about adiponectin's effect and its receptors, with respect to obesity, in dogs. Currently, the relationship between adiposity and adiponectin has yet to be unequivocally demonstrated (German, 2012; Verkest et al., 2012). In our study, as compared to healthy lean counterparts, overweight Miniature Dachshund demonstrated significantly reduced plasma adiponectin concentrations. Interestingly though, qRT-PCR revealed a significant overall down regulation in ADIPOR1 mRNA expression of overweight as compared to healthy lean animal PBL. The expression of ADIPOR1/R2 *in vivo* has been purported to inversely correlate with plasma insulin levels (Leth et al., 2008), with insulin negatively regulating the expression levels of adiponectin receptors and insulin sensitivity in muscle and adipose according to mice studies (Tsuchida et al., 2004). Although, plasma insulin was not significantly higher in overweight dogs in this study, there was a tendency for greater circulating levels which may have had an influence.

Dysregulation of energy metabolism in overweight and obese dogs was further validated by qRT-PCR results observed with FAS, G6PDH and MDH. MDH is an enzyme involved in gluconeogenesis, with a key role to play in the citric acid cycle.

MDH and G6PDH mRNA expression were higher in overweight as opposed to healthy lean counterparts, indirectly indicating greater levels of glucose and lipid metabolism in overweight animals. However, FAS mRNA expression in overweight dog PBL was reduced in overweight as opposed to healthy lean counterparts. FAS is involved in fatty acid synthesis and has been shown to be reduced in high fat feeding studies involving mice and cats (Kim et al., 2003; Lee et al., 2011).

# Conclusion

Leukocytes continually interact with virtually every organ and tissue in the whole body. Therefore, the gene expression responses of circulating PBL can potentially provide early warning of any abnormalities they discover (Shen et al., 2007; Visvikis-Siest et al., 2007; Halvatsiotis et al., 2010). Overall, alterations to insulin signaling (IRS-1, IRS-2, PI3-K), adiponectin signaling (ADIPOR1, ADIPOR2), energy homeostasis (G6PDH, MDH), and lipid metabolism (FAS) gene expression were detected using qRT-PCR in PBL of overweight Miniature Dachshund as compared to lean counterpart PBL. Nonetheless, the majority of the PBL altered mRNA expression trends corroborated with many other studies focusing on obesity influenced mRNA expression trends in tissues such as adipose, muscle, or liver. As such, the use of PBL may hold promise to indirectly gauge for changes, occurring with insulin and adiponectin signaling genes, which may serve as predictive biomarkers of obesity in dogs.

# Tables and Figures

**Table 1 Primer sequences for quantitative real-time PCR**

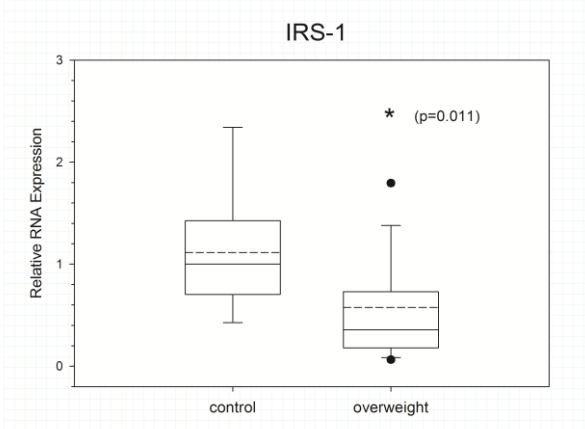
| Probe | PCR product length (bp) | Primer type | Primer Sequences (5'-3') | GenBank Acc.No. |
|---|---|---|---|---|
| IRS-1 | 81 | Forward<br>Reverse | acctgcgttcaaggaggtctg<br>cggtagatgccaatcaggttc | XM_543274 |
| IRS-2 | 177 | Forward<br>Reverse | tggcaggtgaacctgaagc<br>gaagaagaagctgtccgagtgg | XM_542667 |
| PI3K P85α | 132 | Forward<br>Reverse | gcattaaaccagacctcattcagc<br>gcgagtattggtcttcagtgttctc | AB436616 |
| ADIPOR1 | 247 | Forward<br>Reverse | cttctactgctccccacagc<br>catcacagccatgaggaaga | XM843263 |
| ADIPOR2 | 146 | Forward<br>Reverse | tccacaaccttgcttcatct<br>tgattccactcagaccaagg | XM534929 |
| MDH | 82 | Forward<br>Reverse | ggtgcagccttggagaaatatg<br>cagtcaggcagttggtattgg | XM_531844 |
| G6PDH | 139 | Forward<br>Reverse | gctacttcgatgaatttgggatc<br>cactttaacaccttgaccttctcg | XM_538209 |
| FAS | 151 | Forward<br>Reverse | tactggaggggccagtgcatca<br>gtcccgagatggtcactgtgtc | AB436619 |
| 18S | 151 | Forward<br>Reverse | gtaacccgttgaaccccatt<br>ccatccaatcggtagtagcg | NW_003729148.1 |

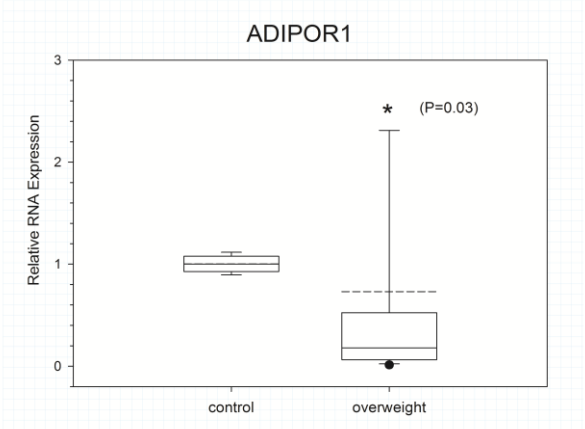
Primer sequences used in quantitative RT-PCR analysis of peripheral blood leukocyte mRNA from lean and overweight dogs. IRS, insulin receptor substrates; ADIPOR, adiponectin receptors; PI3-K, phosphatidylinositol-3 kinase; FAS, fatty acid synthase; G6DPH, glucose-6-phosphate dehydrogenase; MDH, malate dehydrogenase; 18s, 18s ribosome RNA

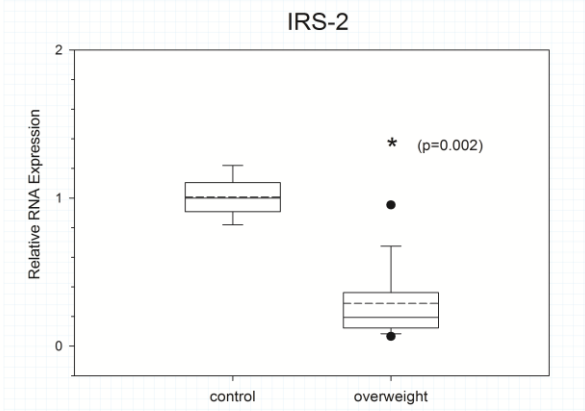
**Table 2 Clinical characteristics and plasma metabolite concentrations**

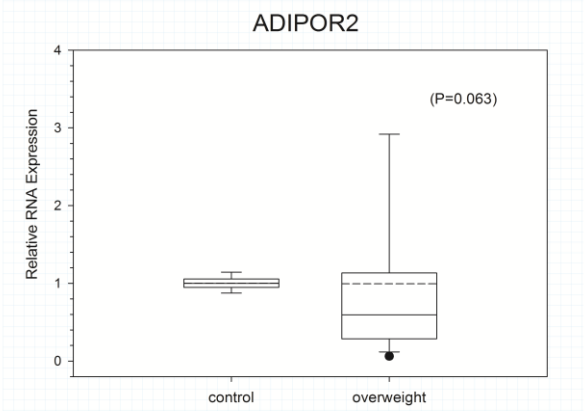
| | Parameter | Miniature Daschunds | |
|---|---|---|---|
| | | Lean (n=3) | Overweight (n=6) |
| Physical Indexes | Age (years) | 7.7 ± 0.6 | 6.5 ± 1.6 |
| | Body Condition Score (1-5) | 3.0 ± 0.0 | 3.8 ± 0.4 |
| | Body Weight (Kg) | 5.1 ± 1.0 | 5.9 ± 1.8 |
| | Amount Body Fat (%) | 23.7 ± 3.2 | 35.7 ± 2.6* |
| | Pelvic Circumference (cm) | 28.0 ± 3.8 | 33.3 ± 4.8** |
| | Hock to Stifle Joint Length (cm) | 10.7 ± 3.1 | 8.6 ± 1.5 |
| DM | Glucose (mg/dl) | 96.7 ± 10.1 | 100.2 ± 25.1 |
| | Insulin (ng/ml) | 0.42 ± 0.26 | 1.30 ± 0.88 |
| Obesity | Adiponectin (mg/ml) | 60.83 ± 18.28 | 24.98 ± 15.46* |
| | Non-estrified fatty acids (mEq/l) | 0.37 ± 0.09 | 0.92 ± 0.33* |
| | Total Cholesterol (mg/dl) | 150.67 ± 24.11 | 239.0 ± 72.18* |
| | Triglycerides (mg/dl) | 32.0 ± 8.54 | 76.67 ± 47.41* |
| Hepatic and Renal Injury | Alanine aminotransferase (U/l) | 34.0 ± 4.58 | 79.60 ± 38.84* |
| | Alkaline Phosphatase (U/l) | 107.67 ± 18.77 | 149.0 ± 69.72 |
| | Aspartate aminotransferase (U/l) | 34.23 ± 10.14 | 73.33 ± 96.41 |
| | Blood Urea Nitrogen (mg/dl) | 14.07 ± 1.27 | 9.98 ± 2.87 |
| | Creatinine (mg/dl) | 0.64 ± 0.14 | 0.49 ± 0.18 |
| | Lactate dehydrogenase (U/l) | 121.33 ± 14.98 | 211.17 ± 233.11 |
| | Total Protein (g/dl) | 6.40 ± 0.26 | 7.00 ± 0.84 |

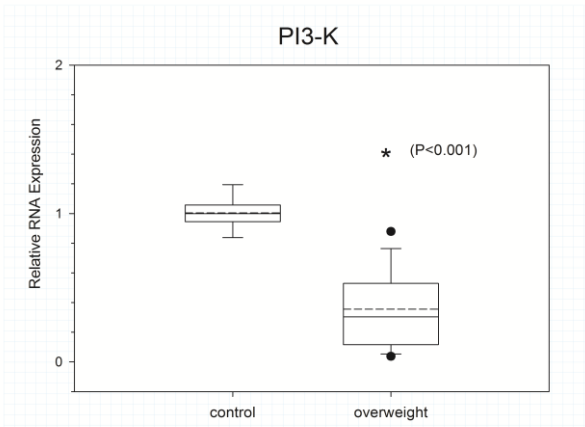
Details of clinical parameters and plasma metabolites monitoring for diabetes mellitus (DM) and obesity, and enzymic assessment of hepatic function in lean overweght dogs. Values presented as mean ± SD. * Significance compared with lean dogs (Mann-Whitney U-test, $P < 0.05$), ** tendency compared with lean dogs ($p<0.1$, Mann-Whitney U-test).

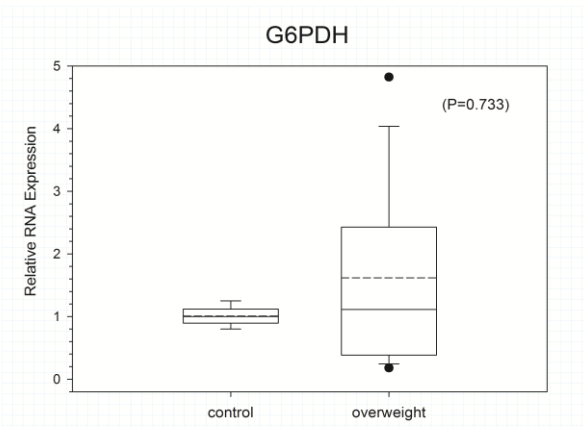

IRS-1
Relative RNA Expression
3
2
1
0
*
(p=0.011)
control
overweight

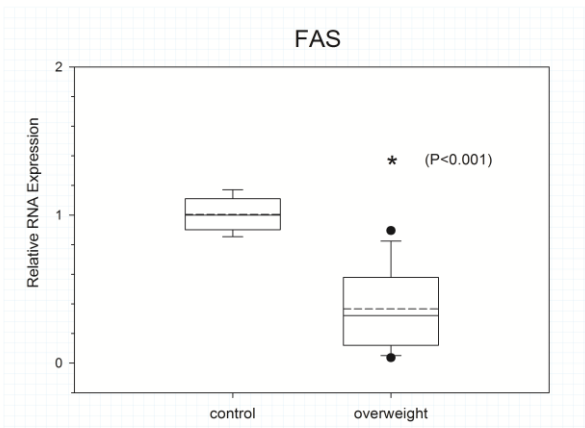

ADIPOR1
Relative RNA Expression
3
2
1
0
*
(P=0.03)
control
overweight


IRS-2
Relative RNA Expression
2
1
0
*
(p=0.002)
control
overweight


ADIPOR2
Relative RNA Expression
4
3
2
1
0
(P=0.063)
control
overweight


PI3-K
Relative RNA Expression
2
1
0
*
(P<0.001)
control
overweight


G6PDH
Relative RNA Expression
5
4
3
2
1
0
(P=0.733)
control
overweight


FAS
Relative RNA Expression
2
1
0
*
(P<0.001)
control
overweight

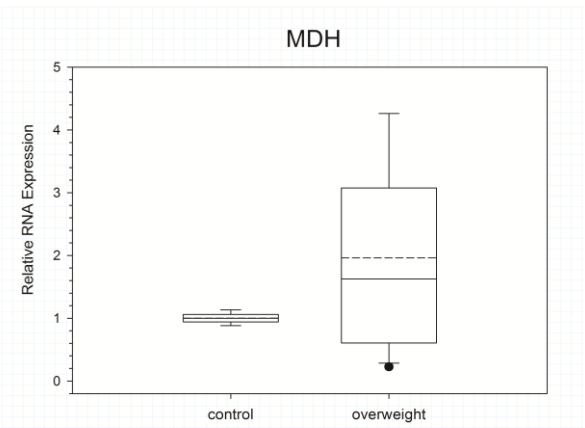

MDH
Relative RNA Expression
5
4
3
2
1
0
control
overweight

**Figure 1.** Comparison of gene expression in peripheral blood leukocytes of overweight and lean healthy Miniature Dachshund. Results are expressed as vertical box plots indicating relative mean mRNA levels in arbitrary units with 10th, 25th, 75th, 90th percentiles and outliers. Median is indicated as a dotted line with The Mann-Whitney *U* Test was used to assess significance between groups, set at $P < 0.05$. Asterisk denotes significantly lower when compared against corresponding lean group. ADIPOR, Adiponectin Receptor; FAS, fatty acid synthase; G6DPH, glucose-6-phosphate dehydrogenase; IRS, insulin receptor substrates; MDH, malate dehydrogenase; PI3-K, phosphatidylinositol-3 kinase.

# CHAPTER Ⅳ

# Gene expression of PBL and insulin sensitive tissues in cats with high-fat diet

# Introduction

Cats have recently been proposed as a valuable animal model for studying human obesity. In order to explore glucose and lipid metabolism in obese cats, it is important to develop molecular tools to investigate transcriptional changes which may be occurring in insulin sensitive tissues. Alterations to gene expression may be a good indicator of metabolic changes occurring in the body (de Godoy et al., 2013). Unfortunately, tissue sampling is a limitation, in particular with human and companion animal genetic studies. As such, peripheral blood leukocytes (PBL) have been purported to be a convenient and readily available source of cells for sampling, with multiple studies reporting that PBL can potentially provide early warning of obesity related disorders in cats and humans (de Mello et al., 2008; Lee et al., 2011; Kollias et al., 2011; Lee et al., 2013). However, there is a lack of studies examining and determining the concordance of gene expression trends in PBL with those found in other tissues in cats.

The objective of this study was to determine whether PBL can serve as an easily accessible cell type for possibly detecting obesity and subsequent obesity risk in cats. In order to meet this objective, mRNA expression profiles of insulin signaling (IRS-1, IRS-2 and PI3-K p85 α), adiponectin signaling (ADIPOR1 and ADIPOR2), energy homoeostasis (G6PDH and MDH), and sterol metabolism (FAS) genes will be compared and contrasted between various insulin sensitive tissues (liver, skeletal muscle, subcutaneous fat, visceral fat and PBL) from cats fed on a high fat (HF) diet.

# Materials and Methods

## Animals and diet

Ten intact, unrelated, cross-bred female cats (1-2 years old) were used for our study. The cats were determined to be at optimal weight and diagnosed to be healthy without any clinical manifestations as determined by veterinarians. All cats were individually housed and maintained for up to two months (eight weeks) at AQS Co. Ltd. (Narita, Japan).

Cats were randomly divided into two groups of 5 animals. One group was designated normal diet, while the other one was designated high fat diet. During the examination period, the normal diet group was fed on a commercial diet (Zoo animal diets ZN for cats, Oriental Yeast Co. LTD., Tokyo, Japan), whereas the high fat diet group was fed on a custom made to order high-fat diet (Nippon Pet Food, Inc., Tokyo, Japan). The composition of both the commercial and high fat diets is shown in Table 1.Coincidentally, prior to this study, all cats were consuming the same commercial diet (Zoo animal diets ZN for cats, Oriental Yeast Co., LTD., Tokyo. Japan). Both groups were fed on their diets ad libitum for their daily energy requirement (DER) from 9:00 AM to 8:30 AM of the next day, for a period of 8 weeks. RER is the resting energy requirement for each cat on the basis of its body weight (BW) before the meal at 9:00 AM, and is calculated as 1.4 × RER (BW0.75 × 70).

Cats were housed in individual cages and provided with water ad libitum. The animal room was maintained at 24 ± 2°C and at 55 ± 10% relative humidity on a 12:12 h light: dark cycle (light on 8:00 AM to 8:00 PM). Living conditions for the

cats were similar before, during, and after our experiment.

**Blood sampling and collection of tissue samples**

At the conclusion of the 8 week feeding schedule, fasted blood (5 ml) was withdrawn from the jugular vein of cats into heparinized tubes. Any surplus diet food was removed at 4:00 PM of the previous day to starve the animals to ensure fasted blood collection. Out of the 5ml of fasted blood collected, 3mL was deposited into PAXgene Blood RNA V.2 kit tubes (PreAnalytiX GmbH) for RNA stabilization, preservation, and sample transport. Tubes were inverted ten times, maintained at room temperature for 2 h, frozen at -20$^{o}$C overnight, and subsequently moved to -80$^{o}$C for storage until further use. The remainder of the blood was collected into heparinized plastic tubes, for immediate centrifugation at 1700 g for 10 min at 4°C to obtain plasma, which was immediately stored at -80°C until required.

Regarding tissue sample collection, 2 cats were randomly chosen from each group (normal and high fat diet) mainly due to ethical reasons as allowed by the University Research Animal Care and Ethics Committee. The animals were fasted overnight and tranquilized with 0.05 mg/kg BW of acepromazine malate (Tech America, KS, US), before being anesthetized with isoflurane. Liver, muscle and adipose tissues samples (2-3 g) were collected and removed from anesthetized animals by laparotomy and all procedures were performed under minimal stress conditions to the animals. Visceral fat was collected from near the jejunum, subcutaneous fat was collected from the inguinal area, and skeletal muscle was

collected from the biceps femoris muscle. Samples were flash-frozen in liquid nitrogen and stored on dry ice until being transferred to -80°C where they were stored until further analysis.

**Plasma metabolite and hepatic enzyme analysis**

Plasma glucose, blood urea nitrogen (BUN), creatinine (CRE), total cholesterol (T-Cho), total protein (TP), and triglyceride (TG) concentrations, as well as lactate dehydrogenase (LDH), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activities, plasma nonesterified fatty acid (NEFA), adiponectin and immunoreactive insulin (IRI) concentrations were determined as previously described in Chapter I.

**Quantitative real-time PCR analysis of tissue and PBL mRNA**

Total leukocyte RNA from blood samples was extracted and isolated using a PAX gene Blood RNA V.2 kit (Qiagen, Düsseldorf, Germany) and a QIAamp RNA Blood Mini Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. Total RNA from liver and muscle samples was extracted by homogenization of liver and muscle samples (50–150 mg) in TRIZOL reagent (Invitrogen, Tokyo, Japan). Total RNA from adipose tissue was extracted and isolated by RNeasy Lipid Tissue Mini Kit (Qiagen, Düsseldorf, Germany)according to the manufacturer's instructions.RNA concentration was assessed by using a Malcom ES-2 (e-spect) micro UV-VIS fluorescence spectrophotometer (Tokyo, Japan). Total RNA

(1 μg) was reverse-transcribed a QuantiTect Reverse Transcription kit (Qiagen, Düsseldorf, Germany) after inactivation of reverse transcription by heating at 95°C for 3 min. The cDNA product was subjected to real-time PCR according to the user instructions for the Real-Time PCR System 7300 (Applied Biosystems, Foster City, CA). qRT-PCR was performed at 95°C for 5 s and 60°C for34 s in 20 μl buffer containing SYBR premix ExTaq II and ROX Reference Dye (Takara Bio, Shiga, Japan) and 0.2 μM each of the primers (Table 2). Absolute quantification, using the standard curve method by establishing a linear amplification curve from serial dilutions of plasmid DNA containing each cDNA, was performed to analyze RT-PCR results. Expression levels of studied genes were normalized to the expression of β-actin, a normal housekeeping gene by expressing a ratio of test gene copy number/ β-actin copy number.

**Statistical analysis**

Plasma metabolite values were expressed as mean ± SD values, and the Student's t-test was used to assess significance between groups, set at $P< 0.05$.qRT-PCR expression values were expressed as median with minimum and maximum values. The Mann-Whitney U-Test was used to assess significance between groups, set at $P< 0.05$. Analysis was performed using SigmaPlot (Version. 11.2, Build 11.2.0.5, Systat Software Inc., San Diego, CA).

# Results

## Analysis of plasma metabolites and hepatic enzymes

Clinical characteristics, general plasma metabolites, and hepatic enzymes of both animal groups are presented in Table 3. High-fat diet feeding resulted in a significant increase ($P<0.05$, Student's t-test) in body weight as compared to control cats only. None of the other plasma metabolites or hepatic enzymes concentrations in high fat diet fed animals significantly differed from control cats.

## Quantitative RT-PCR gene expression profile between control and high-fat diet fed cats in various insulin sensitive tissues

Comparison of different insulin sensitive tissues gene expression trends at the mRNA level, between high-fat diet and control cats are presented in Figure 1. With regards to insulin signaling activity and glucose metabolism, high-fat diet cats had a significantly lower ($p<0.05$, Mann-Whitney U-Test) IRS-1 mRNA level in abdominal fat and peripheral leukocytes as compared to control cats. However, high-fat diet cats had a significantly increased IRS-1 mRNA level in liver as compared to control cats. High-fat diet fed cats' subcutaneous and visceral fat demonstrated a significant ($p<0.05$, Mann-Whitney U-test) median reduction in IRS-2 mRNA expression with a tendency($p<0.10$, Mann-Whitney U-test) for increased IRS-2 expression in high-fat diet cats' liver when compared against controlcats.PI3K p85αmRNA expression was significantly ($p<0.05$, Mann-Whitney U-test) increased in liver and skeletal muscle, significantly ($p<0.05$, Mann-Whitney U-test) reduced in PBL, and tended ($p<0.10$,

Mann-Whitney U-test) to increase in subcutaneous adipose of high-fat diet cats when compared against control cats.

With respect to lipid synthesis and adiponectin signaling, high-fat diet cats' abdominal adipose demonstrated a significant ($p<0.05$, Mann-Whitney U-test) median increase in ADIPOR1mRNA expression, as compared to control cats. In contrast, significantly lower levels ($p<0.05$, Mann-Whitney U-test) of ADIPOR1 mRNA expression in liver and PBL in high-fat diet cats. High-fat diet cats subcutaneous and visceral adipose showed a significant ($p<0.05$, Mann-Whitney U-test) median increase in ADIPOR2 mRNA expression as compared to control cats. There was also a tendency ($p<0.10$, Mann-Whitney U-test) for reduced ADIPOR2 mRNA expression in PBL of high-fat cats. High-fat diet cats FAS mRNA expression was significantly higher ($p<0.05$, Mann-Whitney U-test) in all the tissues except PBL than control cats. In addition, high-fat diet cats G6PHD mRNA expression was significantly higher ($p<0.05$, Mann-Whitney U-test) in liver and skeletal muscle, but significantly ($p<0.05$, Mann-Whitney U-test) lower in PBL as compared to control cats,

Lastly, regarding energy homeostasis, high-fat diet cats abdominal and subcutaneous adipose demonstrated a significant ($p<0.05$, Mann-Whitney U-test) median increase, while liver and PBL demonstrated a significant $p<0.05$, Mann-Whitney U-test) reduction in MDH mRNA expression as compared to control cats.

## Discussion

In our study, when comparing plasma metabolite profiles of HF diet and control cats, HF diet cats demonstrated only a significant increase in body weight (~35% greater) without being accompanied by any significant alterations to biochemical parameters commonly associated with obesity risk, such as NEFA, TG, total cholesterol, insulin, or glucose concentration, when compared to control cats, One possible explanation for the lack of significant alterations to biochemical parameters is that daytime restricted feeding (eg. food is provided ad libitum for 3-5 h at the same time every day, usually during daytime) can attenuate the disruptive effect that diet-induced obesity has on circadian expression of metabolic factors (Sherman et al., 2012). Disruption of circadian rhythms can lead to obesity and metabolic disorders. High-fat feeding modifies behavioral and molecular circadian rhythms in mice leading to metabolic abnormalities mimicking the human metabolic syndrome, including obesity and insulin resistance (Kohsaka et al., 2007; Mendoza et al., 2008). To counter this, timed restricted feeding provides a time cue and resets the circadian clock, leading to better health. Sherman et al. (Sherman et al., 2012) demonstrated that a timed restricted feeding HF diet leads to increased insulin sensitivity and fat oxidation and decreased body weight, fat profile, and inflammation contrary to HF-diet-fed mice but comparable to low fat (LF)-diet-fed mice.

Another explanation for a lack of perturbations observed in plasma metabolites of HF diet cats is that cats used in our study were all intact and did not under gonadectomies. Backus et al. (2007) provided evidence that intact animals, as

opposed to those who underwent gonadectomies, seem more resilient to gain body weight and a congruent increase in insulin due to a high fat diet. Only when a threshold level was met, did intact cats given the highest-fat diet (fat = 64% of metabolisable energy (ME) in a purified diet of constant protein: ME ratio) gain a significant amount of body weight (17% ± 5% greater). Nguyen et al. (Nguyen et al., 2004) also observed similar results between intact and neutered cats. As such, intact animals may be more resilient to high-fat diet induced serum perturbations.

Regarding PBL concordance, using a high-fat diet induced obesity cat model, 8 genes were examined, concordance was observed with ~60% (5 out of 8 ) of them (IRS-1, IRS-2, Adipo-R1, Adipo-R2, and MDH) between PBL and tissue transcriptomes in HF diet fed cats. HF diet cat PBL IRS-1 and IRS-2 mRNA expression were both reduced, when compared to control diet, which was in concordance with reduced IRS-1 and IRS-2 mRNA expression in both abdominal and subcutaneous adipose of HF diet cats. Similar to IRS-1 and IRS-2, ADIPOR1 and ADIPOR2 mRNA expression in HF diet cats was also reduced, when compared to control diet, which was in concordance with reduced ADIPOR1 and ADIPOR2 in liver and skeletal muscle, respectively of HF diet cats. Lastly, PBL MDH mRNA expression was reduced, and was concordant with reduced mRNA expression in liver and skeletal muscle. Surprisingly however, the remaining three genes in which no concordance in mRNA expression was observed (PI3-K, G6DPH, and FAS) between PBL and any of the sampled tissues demonstrated very uniform and polar expression trends between PBL and tissues. For example, all tissues (liver, skeletal muscle,

abdominal omental and subcutaneous adipose) demonstrated increased PI3-K, G6DPH, and FAS mRNA expression trends in HF diet, as compared to diet control cats. Alternately, HF diet fed cats PBL demonstrated reduced mRNA expression trends for the aforementioned genes. Therefore, overall, PBL sensitivity to a high fat diet appears great enough to lead to some degree of transcriptome concordance with insulin sensitive tissues.

In our study, the concordance observed tended to more likely occur with skeletal muscle and liver as opposed to adipose (4 out of 5 concordant genes) which would suggest for a possible bias in tissue representation perhaps, depending on disease or pathological condition. For example, two other diet induced obese studies were conducted by our laboratory, whereby control and obese animals were fed on the same balanced diet, except that obese animals were given 2x their daily RER for up to 6 weeks, in order to see the effect of obesity, and not diet perse; PBL transcriptome concordance was significantly higher with tissues (5 out of 5; IRS-1, IRS-2, PI3-K, MDH, G6DPH;) (8 out of 8 genes; IRS-1, IRS-2, PI3-K, MDH, G6DPH, SREP1-c, FAS, and ACL; [unpublished data]. The higher level of concordance in the other studies may have been attributed to the fact that the influence of obesity and not diet was key since animals were receiving more energy than required, whereas in this study, animals were receiving greater amounts of dietary fat in their diet, making up a greater portion of their caloric intake which was still regulated to their daily RER. As such, PBL sensitivity to obesity versus diet induced alterations in tissue may be greater, thereby leading to a more uniform concordance in transcriptome trends.

However, the more important question which begs our attention is what implication would PBL transcriptome concordance have if it only represents or reflects what may be happening in certain tissues and not others?

The lack of uniformity in gene expression pattern between tissues is a given and expected, since different insulin sensitive tissues accordingly respond differently from one another regarding diet induced obesity (Lee et al., 2012). The response of individual genes to obesity is distinct and largely tissue specific, and a systems approach shows numerous commonly activated pathways, suggesting a coordinated attempt by tissues to limit metabolic perturbations occurring in early-stage obesity. Therefore, tissues showing commonly activated pathways would be more likely to show transcriptome concordance, as opposed to others lacking them. As such, for example, some mRNA expression trends were more likely to be similar between liver and skeletal muscle (IRS-1, IRS-2, MDH) as opposed to abdominal and omental adipose (ADIPOR1 and ADIPOR2) in our study. Alternately, some mRNA expression trends were uniform across all tissues examined (PI3-K, G6DPH, and FAS). Therefore, any concordance observed between PBL and tissue transcriptomes needs to be cautiously interpreted.

This study had a number of limitations that should be considered. First, the HF diet induced model of obesity used in this study does not appear to be sufficiently robust enough to clearly allow for a clear distinction between a diet effect versus an obesity effect. Our high-fat diet induced obesity model required cats to endure an extended 8 week feeding trial using a high fat (HF) diet (24% vs 16% of total weight)

capable of providing 10% greater calories (4660 vs 4210 kcal/kg food) as compared to the control diet given to control animals. However, although the cats had *ad libitum* access to food, the amount of food given to them was regulated according to a capped caloric intake as calculated according to their individual daily energy requirements. So although HF diet cats were receiving a greater percentage of calories from fat, their overall caloric intake was not greater than that of control animals. Therefore, if HF diet cats were to develop obesity, it would not necessarily be due to an excess of caloric intake which is the traditional method of obesity. Instead it might be attributed to the long term consumption of higher dietary fat content. The impact of our HF diet was not very pronounced, with no remarkable alterations to plasma metabolites commonly associated with obesity risk in HF diet fed animals. For the study design to be optimal, both groups of cats should have received the same diet (control or high fat), with one group fed to maintain stable weight, and the other group overfed to develop obesity by excess caloric intake. In this way, animals fed on control or high fat diets would allow us to clearly see obesity and a diet effect, respectively. We will pursue this design in future studies. Second, because two different groups of animals, and not the same animals before and after weight gain were used, the high fat diet induced obesity effect is actually being applied on a different set of animals than the ones we are comparing against. Therefore without a proper baseline established, we cannot preclude the possibility that the same animals, before and after weight gain, would have shown more significant differences in plasma metabolites as opposed to the lack of differences in the studied analyses observed in our study when comparing

control and HF diet groups. In future diet induced obesity studies, we will collect and compare plasma analyses values of the same HF diet animals before and after weight gain for better accuracy, when determining changes occurring in plasma metabolite profile due to diet induced obesity. Third, this was a preliminary study and due to ethical reasons, the tissue group size was small (n=2 for control and HF Diet and could not be increased. This small number reduced the power of the study and may have precluded other significant differences from emerging with respect to PBL profiles between control and HF diet cats, in addition to PBL transcriptome concordance patterns with tissues. In addition, we acknowledge that caution is required with our results since any study in the future with a much larger sample group may not render similar trends or patterns observed in our study. We hope to be able to increase the numbers of samples in future studies, after receiving clearance from our animal review committee, in order to repeat and validate our results on a larger scale. Lastly, because mRNA expression may change rapidly in tissue and can only provide a snapshot of the metabolic processes occurring at that particular time, mRNA expression trends do not necessarily translate over to the protein level. Therefore, careful interpretation of mRNA expression trends is required taking into account that corresponding protein levels have not been measured.

# Conclusion

Overall, our results demonstrate that PBL can serve to act as surrogate tissue for various insulin sensitive tissues, depending on 1) the genes of interest, 2) the degree of pathology associated with the insulin sensitive tissue, and 3) the disease condition. Unfortunately, the expression pattern of the aforementioned genes examined in this study was not uniform between all the tissues examined, and therefore PBL pattern did not match any one particular tissue resulting from a HF diet.

However, the lack of uniformity in gene expression pattern is a given, since different insulin sensitive tissues accordingly respond differently from one another regarding obesity (Lee et al., 2012). Analyses revealed that the response of individual genes to obesity is distinct and largely tissue specific, and a systems approach shows numerous commonly activated pathways, suggesting a coordinated attempt by tissues to limit metabolic perturbations occurring in early-stage obesity.

# Tables and Figures

**Table 1.** Composition of the experimental diets

| | Control | High-fat diet |
|---|---|---|
| Crude protein (%) | 33.6 | 32.7 |
| Crude fat (%) | 16 | 23.9 |
| Crude fiber (%) | 3.5 | 0.9 |
| Crude ash (%) | 5.5 | 5.5 |
| Moisture (%) | 5.5 | 7 |
| Nitrogen free extract (%) | 35.9 | 29.9 |
| Caloric content (kcal/kg) | 4210 | 4660 |

**Table 2.** Primer sequences for quantitative real-time PCR

| Probe | PCR product length (bp) | Primer type | Primer Sequences (5'-3') | GenBank Acc.No. |
|---|---|---|---|---|
| IRS-1 | 81 | Forward | acctgcgttcaaggaggtctg | XM_543274 |
| | | Reverse | cggtagatgccaatcaggttc | |
| IRS-2 | 177 | Forward | tggcaggtgaacctgaagc | XM_542667 |
| | | Reverse | gaagaagaagctgtccgagtgg | |
| PI3K p85α | 132 | Forward | gcattaaaccagacctcattcagc | AB436616 |
| | | Reverse | gcgagtattggtcttcagtgttctc | |
| ADIPOR1 | 212 | Forward | cttctactgctccccacagc | XM843263 |
| | | Reverse | ccactgtggtggccttga | |
| ADIPOR2 | 102 | Forward | tccacaaccttgcttcatct | XM534929 |
| | | Reverse | cctcgatactgaggggtagc | |
| MDH | 82 | Forward | ggtgcagccttggagaaatatg | XM_531844 |
| | | Reverse | cagtcaggcagttggtattgg | |
| G6PDH | 139 | Forward | gctacttcgatgaatttgggatc | XM_538209 |
| | | Reverse | cactttaacaccttgaccttctcg | |
| FAS | 151 | Forward | tactggaggggccagtgcatca | AB436619 |
| | | Reverse | gtcccgagatggtcactgtgtc | |
| β-Actin | 129 | Forward | gccaaccgtgagaagatgact | AF021873 |
| | | Reverse | cccagagtccatgacaataccag | |

Primer sequences used in quantitative RT-PCR analysis of peripheral blood leukocyte mRNA from lean and overweight dogs. ADIPOR, adiponectin receptors; FAS, fatty acid synthase; G6DPH, glucose-6-phosphate dehydrogenase; IRS, insulin receptor substrates; MDH, malate dehydrogenase; PI3-K, phosphatidylinositol-3 kinase; 18s, 18s ribosmal RNA

**Table 3** Clinical characteristics and plasma metabolite concentrations in cats

| Parameters | Control group (n=5) | High-fat Diet (n=5) |
|---|---|---|
| Age (month) | 12.0±0 | 13.6±1.7 |
| Before study Body Weight (Kg) | 2.4 ± 0.3 | 2.6 ± 0.3 |
| After study Body Weight (Kg) | 2.5 ± 0.25 | 3.4 ± 0.5 * |
| Glucose (mg/dl) | 75.6 ± 3.4 | 84.4 ± 9.2 |
| Insulin (ng/ml) | 1.4 ± 0.2 | 1.6 ± 0.2 |
| Non-estrified fatty acids (mEq/l) | 0.6 ± 0.3 | 0.5 ± 0.2 |
| Adiponectin (mg/ml) | 5.6 ± 2.4 | 6.4 ± 2.2 |
| Triglycerides (mg/dl) | 51.8 ± 7.8 | 51.8 ± 32.4 |
| Total Cholesterol (mg/dl) | 106.4 ± 7.0 | 115.4 ± 27.0 |
| Aspartate aminotransferase (U/l) | 30.8 ± 2.1 | 34 ± 4.1 |
| Alanine aminotransferase (U/l) | 61.8 ± 9.3 | 62 ± 11.5 |
| Alkaline Phosphatase (U/l) | 114.6 ± 36.4 | 134.6 ± 58.8 |
| Blood Urea Nitrogen (mg/dl) | 22.2 ± 1.3 | 22 ± 2.6 |
| Creatinine (mg/dl) | 0.88 ± 0.1 | 1.0 ± 0.2 |
| Lactate dehydrogenase (U/l) | 122.8 ± 33.6 | 120.6 ± 30.1 |
| Total Protein (g/dl) | 6.9 ± 0.4 | 6.8 ± 0.4 |

Values presented as Mean ± SD.

* indicates significance when compared against control cats. ($P<0.05$, Student's t-test).

g

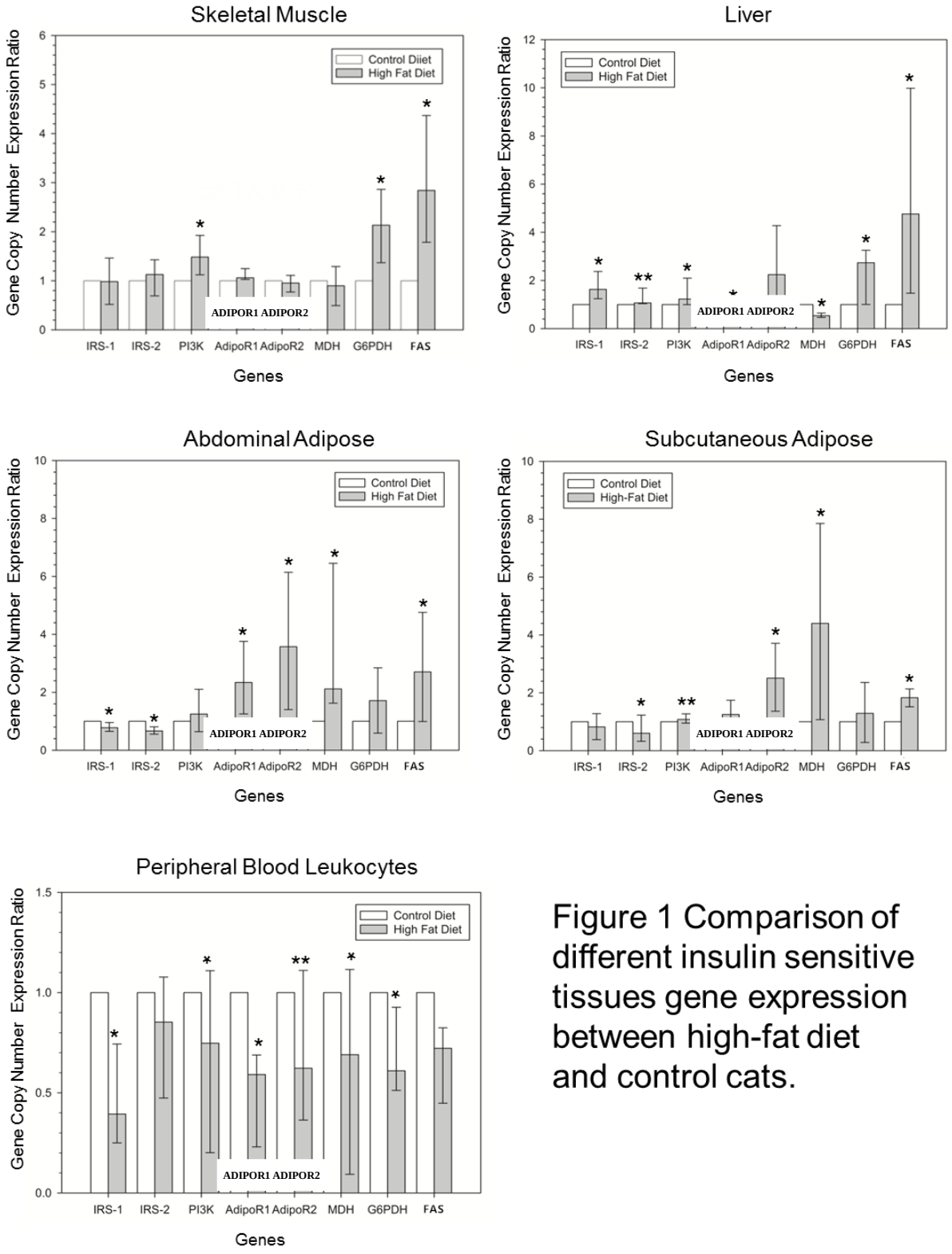



Figure 1 Comparison of different insulin sensitive tissues gene expression between high-fat diet and control cats.

**Figure 1**. Comparison of gene expression in various insulin sensitive tissues (skeletal muscle, liver, abdominal and subcutaneous adipose, and peripheral blood leukocytes) of high-fat diet and control diet fed cat.

Genes examined by RT-PCR include insulin signaling (IRS-1, IRS-2, and PI3-K genes), adiponectin signalling (ADIPOR1 and ADIPOR2), lipid metabolism (FAS and G6PDH), and energy metabolism (MDH) genes. Results expressed as median gene copy number expression ratio. Control cat gene copy number expression ratio = (Control Diet Gene copy number / Control Diet β-actin copy number)/(Control Diet Gene copy number / Control Diet β-actin copy number) and serves to act as a reference for each gene to be compared against by the high-fat diet. High-fat cat gene copy number expression ratio = (High-Fat Diet Gene copy number / High-Fat Diet β-actin copy number)/(Control Diet Gene copy number / Control Diet β-actin copy number) * Significance compared with control diet cats ($p<0.05$, Mann-Whitney U-test), ** Tendency compared with control diet cats ($p<0.10$, Mann-Whitney U-test). Bars indicate upper and lower range values. ADIPOR, adiponectin receptors; FAS, fatty acid synthase; G6PDH, glucose-6-phosphate dehydrogenase; IRS, insulin receptor substrates; MDH, malate dehydrogenase; PI3-K, phosphatidylinositol-3 kinase.

# CHAPTER Ⅴ

# Insulin and adiponection gene expression of PBL in short- and long-term obese cats

# Introduction

Obesity in cats, as in humans, is a predilection risk factor for the development of various chronic diseases (Burkholder et al., 2000). The majority of obese cat studies have been based on experimental short-term dietary manipulation, whereas long-term naturally occurring (NO) obese cats are more markedly overweight, and more representative of the true clinical picture. Therefore, differences between short-term diet induced and long term natural occurring obesity may need to be addressed and resolved, leading to better obesity diagnosis and clinical prevention in cats.

Peripheral blood leukocyte (PBL) transcriptomes have been used for investigating the molecular mechanisms underlying several human diseases (Eady et al., 2005). Moreover, the use of PBL has been advocated for exploring glucose and lipid metabolism in obese cats and dogs (Mori et al., 2009; Li et al., 2012; Lee et al., 2013), especially when a high concordance rate (>80%) of gene expression between PBL and other tissues has been shown in humans and some other species (Liew et al., 2006). Therefore, the aim of this preliminary study in this chapter was to assess changes occurring with plasma metabolite parameters and PBL mRNA transcriptome profiles of genes mainly involved with energy homeostasis (MDH, G6PDH), insulin signaling (IRS-1, IRS-2), adiponectin signaling (ADIPOR1, ADIPOR2), and lipogenesis (FAS) in short-term high-fat diet induced (HFD) and long-term naturally occurring obese cats.

# Materials and Methods

## Animals

Eight intact, unrelated, cross-bred female cats (1–2 years old) served as our lean control and short-term high fat diet induced HFD obesity group. Same experimental cats as previously described in Chapter Ⅳ.

Seven client-owned cross-bred cats (1 female and 6 neutered males; 6-14 years old), which presented themselves at a private veterinary clinic in Tokyo, Japan for vaccination between April and May 2012, were selected to represent our long-term NO obesity group (BCS≥4). None of the selected animals had any evidence of acute or chronic disease (except for overweight/obesity for at least 1 year) based on physical and clinical examination of routine hematologic and biochemical analysis. Owner consent was obtained for all animals used in our study. The body condition score (BCS) of each cat was assessed using the five point scale (Burkholder et al., 2000) by a veterinarian at the clinic, using the amount of fat covering the rib area, as judged by visual inspection and palpation.

## Blood sampling

Blood samples (4 hr postprandial) (4-5ml) were collected from the cephalic vein of lean control and HFD animals without the aid of sedation, prior to (baseline values) and at the conclusion of the 8 week feeding schedule. Blood samples from naturally obese cats were collected in a similar manner, except with not time restrictions. Three ml of this blood was collected into PAXgene Blood RNA V.2 kit

tubes (PreAnalytiX GmbH) for RNA stabilization, preservation, and sample transport. Tubes were inverted ten times, maintained at room temperature for 2 h, frozen at -20 °C overnight and subsequently moved to -80 °C for storage until further use. The remainder of blood was collected into heparinised plastic tubes, for immediate centrifugation at 1200 *g* for 10 min at 4 °C to obtain plasma which was immediately stored at -80 °C until required.

**Plasma metabolite and hepatic enzyme analysis**

Plasma glucose, blood urea nitrogen (BUN), creatinine (CRE), total cholesterol (T-Cho), total protein (TP), and triglyceride (TG) concentrations, as well as lactate dehydrogenase (LDH), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activities, plasma nonesterified fatty acid (NEFA), adiponectin and immunoreactive insulin (IRI) concentrations were determined as previously described in Chapter I.

**Quantitative real-time PCR analysis of peripheral blood leucocyte mRNA**

Total leukocyte RNA and plasma were collected and processed from blood samples (see Chapter Ⅳ material for methods). Clinical characteristics and plasma metabolite values for all groups are provided in Table 1 and expressed as median ± min/max values. Quantitative RT-PCR reactions, using SYBR Green fluorescent dye, for genes of interest (IRS-1, IRS-2, PI3K p85 α, MDH, G6PDH, FAS, ADIPOR1, ADIPOR2) were performed in triplicate, with β-actin serving as internal standards

(see Chapter Ⅳ material for methods). Primers are same with Chapter Ⅳ.

**Statistical analysis**

Expression levels of studied genes were normalized to the expression of β-actin and represented as a ratio of test gene copy number/ β-actin copy number. Data are reported as median ± min/max range. The Mann–Whitney U-Test was used to determine significance for plasma metabolite and qRT-PCR results between groups with statistical significance being set at $P < 0.05$. Tests were conducted using SigmaPlot 11.2 analysis software (Build 11.2.0.5, Systat Software Inc., San Diego, CA).

# Results

## Analysis of plasma metabolites and hepatic enzymes

Clinical characteristics and median values of general plasma metabolites and hepatic enzymes are presented in Table 3. Short-term HFD induced obesity cats resulted in a significant increase ($P<0.05$, Mann-Whitney U-Test) in plasma AST, ALT as compared to lean animals, but significant decrease ($P<0.05$, Mann-Whitney U-Test) in plasma insulin. Long-term NO obesity cats showed that a significant increase ($P<0.05$, Mann-Whitney U-Test) in plasma glucose, NEFA, T-Cho, BUN, CRE and TP as compared to lean animals, but significant decrease ($P<0.05$, Mann-Whitney U-Test) in plasma insulin..

## Quantitative RT-PCR comparative analysis of peripheral blood leukocyte mRNA

Comparison of PBL gene expression trends at the mRNA level, in short-term HFD induced obesity cats, long-term NO obesity cats and lean cats PBL are presented in Figure 1. With regards to insulin signaling activity, short-term HFD induced obesity cats PBL demonstrated a significant ($p<0.05$, Mann-Whitney U-test) median reduction in IRS-1 mRNA expression as compared to lean cats PBL. Long-term NO obesity cats PBL demonstrated a significant ($p<0.05$, Mann-Whitney U-test) median decrease in IRS-1 and IRS-2 mRNA expression, as compared to lean animal PBL. However, no accompanying difference in mRNA expression level was observed with PI3K p85α in both obesity cats PBL.

With respect to lipid synthesis and adiponectin signaling, long-term NO

obesity cats PBL demonstrated a significant ($p<0.05$, Mann-Whitney U-test) median decrease in FAS mRNA expression, as compared to lean cats PBL. Moreover, both obesity group cats displayed significant ($p<0.05$, Mann-Whitney U-test) median decrease in ADIPOR1 and R2 mRNA expression as compared to lean cats PBL. Lastly, regarding energy homeostasis, short-term HFD induced obesity cats PBL demonstrated a significant ($p<0.05$, Mann-Whitney U-test) median increase in MDH mRNA expression, as compared to lean animal PBL.

## Discussion

Short-term HFD cats demonstrated a significant increase in AST, and ALT as compared to lean animals. Raised circulating levels of AST and ALT are considered to be indicators of liver disease, and this observation is consistent with the idea of high-fat diet induced obesity resulting in an increased accumulation of fat in the liver over a short time (Yang et al., 2012). Alternately, NO obese cats showed dysregulation of lipid metabolism and chronic liver and kidney injury (Mori et al., 2012), as demonstrated by a significant increase in NEFA, T-Cho, BUN, CRE and TP as compared to lean animals. In addition, there was a trend for increased TG in NO obese animals also. The period of time cats are exposed to obesity can significantly affect metabolic parameters.

Although differences in plasma lipid metabolite levels was observed between HFD and NO groups, PBL demonstrated a significant median decrease in FAS mRNA expression for the NO group, and with a tendency to decrease in the NO group as compared to lean cats PBL. FAS is involved in fatty acid synthesis and due to the large amount of plasma NEFA (either circulating due to long term obesity or constant ingestion via HF diet), tissues need not synthesize fatty acids, absorbing them from circulation instead. Regarding energy homeostasis, MDH is an enzyme involved in gluconeogeneis, with a key role to play in the citric acid cycle. HFD cat PBL demonstrated a significant median increase in MDH mRNA expression, as compared to lean animal PBL. Although not statistically significant, NO cat PBL demonstrated a trend for increased MDH mRNA expression also. G6PDH supplies reducing energy to

cells by maintaining the level of NADPH, which can be used by tissues actively engaged in biosynthesis of fatty acids and/or isoprenoids. Although no statistical significance in PBL G6PDH mRNA expression between both groups and lean animals was observed, there was a tendency for higher G6PDH mRNA expression in NO cat PBL. Overall, both HFD and NO cats appear to be experiencing greater levels of glucose and lipid metabolism due to obesity in general, and especially due to a high fat diet in HFD cats. However, the trend in increased G6PDH mRNA expression of NO cat PBL may be indicative of real location of lipid stores throughout the body.

Interestingly however, both HFD and NO obese animals had significantly lower circulating insulin levels as compared to lean animals, but only NO obese animals had a corresponding significant increase in amount of circulating glucose. This might indicate possible β-cell function impairment and insulin resistance in NO animals since post-prandial glucose levels remain high. Obesity can induce insulin resistance by reducing the responsiveness to insulin via insulin receptor and post-receptor defects in glucose metabolism (Mori et al., 2009) HFD and NO cat PBL demonstrated a significant ($p<0.05$, Mann-Whitney U-test) median reduction in IRS-1 and both IRS-1 and IRS-2 mRNA expression, respectively, as compared to lean animals. IRS-2 mRNA expression plays a critical role in regulation of β-cell growth and decreased IRS-2 expression causes spontaneous β-cell apoptosis (Withers et al., 1998), which may help to explain the greater severity of insulin resistance in NO cats. However, no accompanying significant difference in mRNA expression level was observed with PI3K p85α in either type of obese cat PBL. However, there was a

tendency for increased expression in HFD cat PBL, thus indicating a possible compensatory mechanism regarding insulin signaling due to reduced circulating insulin, hence leading to reduced circulating insulin but normal glucose levels observed in plasma.

A reduction of adiponectin can also play a causal role in the development of insulin resistance. Adiponectin can play a role in insulin synthesization and acts through two cell membrane receptors, ADIPOR1 and R2 (Kadowaki et al., 2006). Although circulating adiponectin concentrations were not affected in either group, a significant median reduction in ADIPOR1 and R2 mRNA expression was observed in PBL of both groups as compared to lean cats. This finding concurs with a human study reporting on decreased surface expression of adiponectin receptors (R1 and R2) in peripheral monocytes of human obese groups as compared to normal-weight controls (Kolias et al., 2011). This might indicate that there is reduced insulin sensitization due to obesity associated down-regulation of adiponectin receptor expression in tissues perhaps in both groups.

# Conclusion

In conclusion, differences in the type of aberrant in plasma metabolite profile were observed between the two different obesity models. However, PBL mRNA transciptome profiles were very consistent regarding expression trends of the genes used in our study highlighting both: 1) the consistent negative effect of obesity, regardless of being acute or long term, on a host; and 2) the sensitivity of PBL to the effects of obesity. Further work on PBL transciptome pattern of additional genes involved with obesity should be pursued in the future.

# Tables and Figures

**Table 1 Clinical characteristics and plasma metabolite concentrations**

| Parameters | Control (n=8) | High-fat food (n=8) | Natural overweight (n=7) |
|---|---|---|---|
| **Age** (months) | 13.0 (10 - 30) | 15.0 (12 - 32) | 71.0 * (10 - 192) |
| **Body Condition Score** (1-5) | 3.0 (2.5 - 3.0) | 4.0 * (3.5 - 4.0) | 4.0 ** (4.0 - 5.0) |
| **Body Weight** (Kg) | 2.4 (2.2 - 3.2) | 3.1 * (2.5 - 4.2) | 5.8 ** (4.3 - 20.0) |
| **Glucose** (mg/dl) | 73.0 (62.0 - 81.0) | 76.0 (66.0 - 99.0) | 136.0 ** (97.0 - 152.0) |
| **Insulin** (ng/ml) | 2.4 (2.1 - 2.7) | 1.6 * (1.4 - 2.0) | 1.65 ** (1.4 - 1.8) |
| **Non-estrified fatty acids** (mEq/l) | 0.4 (0.3 - 0.6) | 0.6 (0.4 - 0.9) | 1.26 ** (0.5 - 2.5) |
| **Adiponectin** (mg/ml) | 4.3 (1.8 - 9.3) | 7.3 (3.7 - 11.2) | 3.0 (1.3 - 7.5) |
| **Triglycerides** (mg/dl) | 47.1 (41.0 - 53.0) | 32.4 (24.0 - 112.0) | 77.0 (22.0 - 631.0) |
| **Total Cholesterol** (mg/dl) | 104.0 (89.0 - 151.0) | 103.0 (70.0 - 158.0) | 143.0 ** (101.0 - 450.0) |
| **Aspartate aminotransferase** (U/l) | 27.5 (22.0 - 35.0) | 32.0 * (30.0 - 41.0) | 31.2 (17.0 - 148.0) |
| **Alanine aminotransferase** (U/l) | 41.0 (35.0 - 73.0) | 70.5 * (43.0 - 79.0) | 52.0 (33.0 - 131.0) |
| **Alkaline Phosphatase** (U/l) | 77.0 (31.0 - 201.0) | 109.5 (73.0 - 245.0) | 99.0 (52.0 - 157.0) |
| **Blood Urea Nitrogen** (mg/dl) | 23.5 (17.0 - 27.0) | 22.5 (18.0 - 26.0) | 28.2 ** (23.0 - 33.0) |
| **Creatinine** (mg/dl) | 0.8 (0.6 - 1.4) | 1.1 (0.8 - 1.2) | 1.6 ** (1.1 - 1.9) |
| **Lactate dehydrogenase** (U/l) | 136.0 (71.0 - 222.0) | 126.5 (77.0 - 285.0) | 173.0 (84.0 - 454.0) |
| **Total Protein** (g/dl) | 6.5 (5.7 - 7.8) | 6.7 (6.0 - 7.5) | 7.5 ** (6.6 - 7.9) |

Values presented as median and range. * High-fat food cats significance compared with control cats, **Natural overweight cats significance compared with control cats (Mann-Whitney U-test, $P < 0.05$).

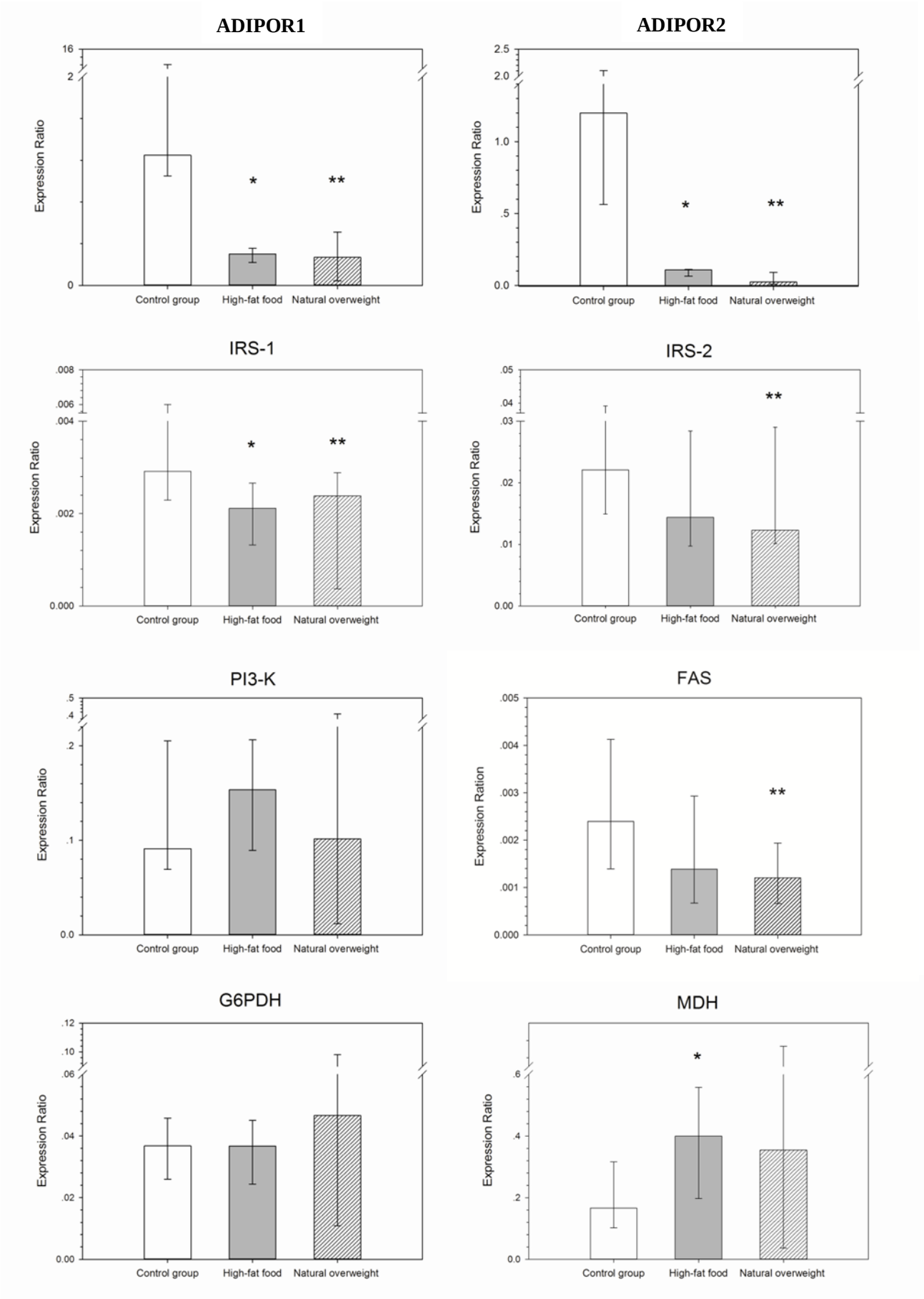

ADIPOR1
Expression Ratio
16
2
0
*
**
Control group
High-fat food
Natural overweight
ADIPOR2
Expression Ratio
2.5
2.0
1.0
.5
0.0
*
**
Control group
High-fat food
Natural overweight
IRS-1
Expression Ratio
.008
.006
.004
.002
0.000
*
**
Control group
High-fat food
Natural overweight
IRS-2
Expression Ratio
.05
.04
.03
.02
.01
0.00
**
Control group
High-fat food
Natural overweight
PI3-K
Expression Ratio
.5
.4
.2
.1
0.0
Control group
High-fat food
Natural overweight
FAS
Expression Ration
.005
.004
.003
.002
.001
0.000
**
Control group
High-fat food
Natural overweight
G6PDH
Expression Ratio
.12
.10
.06
.04
.02
0.00
Control group
High-fat food
Natural overweight
MDH
Expression Ratio
.6
.4
.2
0.0
*
Control group
High-fat food
Natural overweight

Figure 1 PBL transciptome profiles of genes mainly involved with energy homoeostasis (MDH, G6PDH), insulin signaling (IRS-1, IRS-2), adiponectin signaling (ADIPOR1, ADIPOR2), and lipogenesis (FAS) in short-term high-fat diet induced (n=8) and long-term naturally occurring obese (n=7) cats being compared against lean control cats (n=8). Expression levels of studied genes were normalized to the expression of β-actin and represented as a ratio of test gene copy number/ β-actin copy number. Data are reported as median min/max range with values being the mean of three independent experiments. * denotes significantly different ($P < 0.05$, Mann–Whitney U-test) when compared against lean control cats. ** denotes significantly different ($P < 0.05$, Mann–Whitney U-test) when compared against high fat diet (HFD) induced obese cats.

# General Summary

A number of health problems purported to be associated with or exacerbated by obesity in dogs and cats. Early warning signs of energy metabolism dysregulation in overweight animals could be detected by the presence of insulin and adiponectin signaling genes via PBL qRT-PCR method, and may be valuable in early diagnosis. In addition, better understanding of these mechanisms might assist in the prevention of the development of obesity-related metabolic dysfunction or diabetes in obese animals.

1. Using body fat percentage assessing overweight status in dogs

Currently, 5 point body condition scoring (BCS) is commonly used by veterinarians and clinicians to assess adiposity in dogs in Japan. However, assigning a BCS score is subjective in nature, and most clinicians do not score with half points, instead preferring to round off values, thereby rendering less accurate assessments. Therefore, we sought to determine whether assessing body fat percentage (BF%), using simple morphometric measurements, and supplementing it with 5-point BCS can render increased sensitivity for detecting increasing adiposity in overweight small breed dogs via plasma metabolite validation.

Overall, lean BF % range was determined to be 15-20% for non-neutered male dogs, and 15-25% for female (non-spayed/spayed) and neutered male dogs. BCS categorized overweight animals displayed significantly higher levels of non-esterified fatty acids (NEFA; $p=0.005$); whereas significantly higher levels of NEFA ($p=0.006$),

Total cholesterol (T-Cho; p=0.029), and Triglycerides (TG; p=0.001) were observed in BCS+BF % categorized overweight animals as compared to lean animals. The increase in sensitivity, due to BF %, for gauging alterations to plasma metabolite values, may be due to increased correlation strength. BF % positively correlated with plasma insulin (r=0.627, p=0.002), NEFA (r=0.674, p<0.001), T-Cho (r=0.825, p<0.0001), TG (r=0.5823, p<0.005), Blood Urea Nitrogen (r=0.429, p<0.05), creatinine (r=0.490, p=0.021), and Total Protein (r=0.737, p<0.0001) levels which all tend to increase as a result of increasing adiposity.

In conclusion, BF % supplementation to 5 point BCS, appears to increase the likelihood of validating overweight status in small breed dogs, by detecting alterations in plasma metabolite values, especially lipid metabolites, induced as a result of increasing adiposity.

2. Plasma lipoprotein profiles and malondialdehyde in hyperlipidemia dogs

The aim of this Chapter is to compare metabolic parameters, malondialdehyde (MDA) as a lipid oxidation marker, and lipid profiles between dogs with untreated hyperlipidemia and hyperlipidemia with treatment, in order to examine the usefulness of MDA and lipid profiles as diagnostic parameters at early stages of hyperlipidemia. Dog samples were collected from clinics which were separated into three groups: control, untreated hyperlipidemia based on temporally screening, and hyperlipidemia with current anti-hyperlipidemic (statins and fibrates) treatment. TG levels of untreated hyperlipidemia dogs were significantly higher than those of control dogs. ALT levels of

hyperlipidemic dogs with treatment were the highest among three groups. VLDL and LDL of both cholesterol and triglyceride of untreated hyperlipidemia dogs were the highest among three groups. HDL1 levels in triglyceride of hyperlipidemia dogs with treatment were significantly higher than those of control and untreated hyperlipidemia dog. MDA concentrations of untreated hyperlipidemia dogs were significantly higher than those of control and hyperlipidemic dogs with treatment. The results indicated that dogs with untreated hyperlipidemia clearly showed abnormal lipid status, whereas hyperlipidemic dogs under anti-hyperlipidemia treatment showed more normal lipid status suggesting the effectiveness of the therapy. Anti-hyperlipidemics (statins and fibrates) for dogs are also effective in relieving elevated levels of lipids and lipid oxidation. Plasma lipid (triglyceride and cholesterol) profiles and MDA are useful diagnostic tools for identifying early stages of untreatment hyperlipidemia in dogs.

3. Insulin and adiponectin gene expression of PBL in obese Miniature Dachshunds

Peripheral blood leukocytes (PBL) continually interact with virtually every organ and tissue in the whole body. A remarkable concordance (80%) of gene expression profiles between peripheral blood mononuclear cells and different tissues has been previously demonstrated in humans. As such, gene expression responses of circulating PBL can therefore potentially provide early warning of any abnormalities they discover. Alteration to weight, when associated with obesity, has been reported to lead to alterations to PBL gene expression, especially those related to insulin and adiponectin signaling genes.

As such, PBL mRNA expression profiles of genes involved in insulin

signaling (ADIPOR (-1 and 2); IRS (-1 and 2); PI3-K) lipogenesis (FAS) and energy homeostasis (G6PDH; MDH) were carried out on lean Miniature Dachshund and compared against similar profiles of breed and age matched overweight Miniature Dachshund in an attempt to identify possible PBL biomarkers for assessing obesity in dogs.

Overweight status resulted in a significant increase in plasma NEFA, T-Cho, triglycerides and ALT, and a significant decrease in plasma adiponectin, as compared to lean Miniature Dachshund. Overweight dogs PBL demonstrated reduced mRNA expression of IRS-1 and -2; PI3-K, ADIPOR1 and FAS genes.

Overall, these findings suggest that dysregulation of energy metabolism, associated with obesity, in overweight dogs may carry over with alterations in PBL gene expression of genes involved in insulin and sterol metabolism. As such, PBL gene expression profiles may aid in early detection of PBL biomarkers for assessing obesity in dogs.

4. High-fat diet cats gene expression in PBL and insulin sensitive tissues

Alterations to gene expression, especially transcriptional changes, occurring in insulin sensitive tissues, may be a good indicator of metabolic changes occurring in the body. The objective of this Chapter is to determine whether PBL can serve as an easily accessible cell type for possibly detecting obesity and subsequent obesity risk in cats.

Regarding insulin signaling activity, high-fat diet cats had a significantly

reduced IRS-1 mRNA expression in abdominal fat and peripheral leukocytes, with a significantly increased IRS-1 mRNA expression in liver as compared to control cats. Moreover, in high-fat diet cats, a significant reduction in IRS-2 mRNA expression in subcutaneous and visceral fat, and a significant increase in PI3K p85αmRNA expression in liver and skeletal muscle with a significant reduction in PBL was observed as compared against control cats. With respect to lipid synthesis and adiponectin signaling, high-fat diet fed cats' abdominal adipose demonstrated a significant median increase in ADIPOR1 mRNA expression, with reduced ADIPOR1 mRNA expression in liver and PBL being observed as compared to control cats. In addition, subcutaneous and visceral adipose demonstrated a significant median increase in ADIPOR2 mRNA expression, and FAS mRNA expression was significantly higher in all tissues except PBL as compared to control cats. Lastly, in high-fat diet fed cats, G6PHD mRNA expression was significantly higher in liver and skeletal muscle, but significantly lower in PBL as compared to control cats. In addition, abdominal and subcutaneous adipose demonstrated a significant median increase, while liver and PBL demonstrated a significant reduction in MDH mRNA expression as compared to control cats.

Overall, our results demonstrate that PBL can serve to act as surrogate tissue for various insulin sensitive tissues, depending on 1) the genes of interest, 2) the degree of pathology associated with the insulin sensitive tissue, and 3) the disease condition. Although the expression pattern of the aforementioned genes examined was not completely uniform, there was some correlation between PBL and various tissues.

The response to obesity is largely tissue specific with numerous commonly activated pathways suggesting a coordinated attempt by tissues to limit metabolic perturbations occurring in early-stage obesity.

5. Insulin and adiponectin gene expression of PBL in short- and long-term obese cats

Naturally occurring obesity is more representative of the true clinical picture than experimental short-term dietary manipulation in cats. The aim of this preliminary study was to compare plasma metabolite and PBL mRNA transciptome profiles of genes mainly involved with energy homoeostasis, insulin and adiponectin signaling, in short-term high-fat diet induced and long-term naturally occurring obese cats.

Plasma metabolite profiling highlighted the inherent aberrations associated with different types and exposure time of obesity. In addition, PBL transciptome profiles were very consistent regarding the genes used in our study, highlighting the sensitivity of PBL to the effects of obesity regardless of being acute or long term, on a host.

Overall, firstly, present studies have showed that BCS supplementing it with body fat percentage (BF%) provided more accurate assessments for dogs. Secondly, the present investigation indicated that plasma lipid profiles and MDA are most likely useful parameters for identifying early stages of obesity with mild hyperlipidemia in dogs. Thirdly, insulin and adiponectin gene expression responses of circulating PBL can potentially provide early warning of any abnormalities, when associated with obesity in both dogs and cats. Lastly, dogs and cats have been proposed as a valuable

animal model for studying human obesity, especially naturally occurring obesity animals.

# References


Appleton, D.J., Rand, J.S., Sunvold, G.D., 2001. Insulin sensitivity decreases with obesity, and lean cats with low insulin sensitivity are at greatest risk of glucose intolerance with weight gain. Journal of Feline Medicine and Surgery 3:211-218.

Backus, R.C., Cave, N.J., Keisler, D.H., 2007. Gonadectomy and high dietary fat but not high dietary carbohydrate induce gains in body weight and fat of domestic cats. The British Journal of Nutrition 98:641-650.

Bailhache, E., Nguyen, P., Krempf, M., Siliart, B., Magot, T., Ouguerram, K., 2003. Lipoproteins abnormalities in obese insulin-resistant dogs. Metabolism: clinical and experimental 52, 559-564.

Bauer, J.E., 2004. Lipoprotein-mediated transport of dietary and synthesized lipidsand lipid abnormalities of dogs and cats. Journal of the American VeterinaryMedical Association 224, 668–675.

Berg, A.H., Combs, T.P., Du, X., Brownlee, M., Scherer, P.E., 2001. The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. Nature Medicine 7, 947-953.

Bergman, R.N., Van Citters, G.W., Mittelman, S.D., Dea, M.K., Hamilton-Wessler, M., Kim, S.P., Ellmerer, M., 2001. Central role of the adipocyte in the metabolic syndrome. Journal of Investigative Medicine 49, 119-126.

Bilheimer, D.W., Grundy, S.M., Brown, M.S., Goldstein, J.L., 1983. Mevinolinstimulates receptor-mediated clearance of low densitylipoprotein from plasma in familial hypercholesterolemia heterozygoted. Transactions of the Association of American Physicians 96, 1-9.

Bray, G.A., 1993. Fat distribution and body weight. Obesity Research 1, 203-205.

Brunzell, J.D., Bierman, E.L., 1982. Chylomicronemiasyndrome: interaction of genetic and acquired hypertriglyceridemia. Medical Clinics of North America 66, 455-68.

Bjornvad, C.R., Nielsen, D.H., Armstrong, P.J., McEvoy, F., Hoelmkjaer, K.M., Jensen, K.S., Pedersen, G.F., Kristensen, A.T., 2011. Evaluation of a nine-point body condition scoring system in physically inactive pet cats. American Journal of Veterinary Research 72:433-437.

Burkholder, W.J., Toll, P.W., 2000. Obesity. In: Small Animal Clinical Nutrition. 4th edition. Edited by Hand MS, Thatcher CD, Remillard RL, Roudebush P. Topkea: Mark Morris Institute, 401-430.

Burton, B.T., Foster, W.R., Hirsch, J., Van Itallie, T.B., 1985. Health implications of obesity: an NIH Consensus Development Conference. International Journal of Obesity 9:155-170.

Caimari, A., Oliver, P., Rodenburg, W., Keijer, J., Palou, A., 2010. Feeding conditions control the expression of genes involved in sterol metabolism in peripheral blood mononuclear cells of normoweight and diet-induced (cafeteria) obese rats. Journal of Nutritional Biochemistry 21, 1127-1133.

Caimari, A., Oliver, P., Keijer, J, Palou, A., 2010a. Peripheral blood mononuclear cells as a model to study the response of energy homeostasis-related genes to acute changes in feeding conditions. OMICS 14, 129-141.

Cheatham, B., Kahn, C.R., 1995. Insulin action and the insulin signaling network. Endocrine Reviews 16, 117-142.

Chikamune, T., Katamoto, H., Ohashi, F., Shimada, Y., 1995. Serum lipid and lipoprotein concentrations in obese dogs. The Journal of Veterinary Medicine Science 57, 595-598.

de Godoy, M.R., Swanson, K.S., 2013. Nutrigenomics: Using gene expression and molecular biology data to understand pet obesity. Journal of Animal Science 91, 2949-2964.

de Mello, V.D., Kolehmainen, M., Pulkkinen, L., Schwab, U., Mager, U., Laaksonen, D.E., Niskanen, L., Gylling, H., Atalay, M., Rauramaa, R., Uusitupa, M., 2008. Downregulation of genes involved in NFkappaB activation in peripheral blood mononuclear cells after weight loss is associated with the improvement of insulin sensitivity in individuals with the metabolic syndrome: the GENOBIN study. Diabetologia 51:2060-2067.

de Mello, V.D., Kolehmainen, M., Schwab, U., Mager, U., Laaksonen, D.E., Pulkkinen, L., Niskanen, L., Gylling, H., Atalay, M., Rauramaa, R., Uusitupa, M., 2008a. Effect of weight loss on cytokine messenger RNA expression in peripheral blood mononuclear cells of obese subjects with the metabolic syndrome. Metabolism 57, 192-199.

Eady, J.J., Wortley, G.M., Wormstone, Y.M., Hughes, J.C., Astley, S.B., Foxall, R.J., Doleman, J.F., Elliott, R.M. 2005. Variation in gene expression profiles of

peripheral blood mononuclear cells from healthy volunteers. Physiological Genomics 22, 402-411.

Fleeman, L., 2009. Is hyperlipidemia clinically important in dogs? Veterinary Journal 183, 10.

German, A.J., Holden, S.L., Moxham, G.L., Holmes, K.L., Hackett, R.M., Rawlings, J.M., 2006. A simple, reliable tool for owners to assess the body condition of their dog or cat. The Journal of Nutrition.136, 2031-2033.

German, A.J., Hervera, M., Hunter, L., Holden, S.L., Morris, P.J., Biourge, V., Trayhurn, P., 2009. Improvement in insulin resistance and reduction in plasma inflammatory adipokines after weight loss in obese dogs. Domestic Animal Endocrinology 37, 214-226.

German, A.J., 2012. Barking up the wrong tree: What's the deal with obesity, adiponectin and inflammation in dogs? Veterinary Journal 194, 272-273.

Ghanim, H., Aljada, A., Hofmeyer, D., Syed, T., Mohanty, P., Dandona, P., 2004. Circulating mononuclear cells in the obese are in a proinflammatory state. Circulation 110, 1564-1571.

Grant, R.W., Vester Boler, B.M., Ridge, T.K., Graves, T.K., Swanson, K.S., 2011. Adipose tissue transcriptome changes during obesity development in female dogs. Physiological Genomics 43, 295-307.

Gupte, R.S., Floyd, B.C., Kozicky, M., George, S., Ungvari, Z.I., Neito, V., Wolin, M.S., Gupte, S.A., 2009. Synergistic activation of glucose-6-phosphate dehydrogenase and NAD(P)H oxidase by Src kinase elevates superoxide in type 2 diabetic, Zuckerfa/fa, rat liver. Free Radical Biology & Medicine 47:219-228.

Halvatsiotis, I., Tsiotra, P.C., Ikonomidis, I., Kollias, A., Mitrou, P., Maratou, E.,Boutati, E., Lekakis, J., Dimitriadis, G., Economopoulos, T., Kremastinos, D.T., Raptis, S.A., 2010. Genetic variation in the adiponectin receptor 2 (ADIPOR2) gene is associated with coronary artery disease and increased ADIPOR2 expression in peripheral monocytes. Cardiovascular Diabetology 9, 10-21.

Hatano, Y., Mori, N., Asada, M., Mori, A., Yamamoto, I., Muranaka, S., Kojima, M., Kigure, M., Yagishita, M., Sako, T., Arai, T., 2010. Hypertriglyceridemia with increased plasma insulin concentrations in cats. Research in Veterinary Science 88, 458-460.

Henson, M.S., O'Brien, T.D., 2006. Feline models of type 2 diabetes mellitus. ILAR Journal 47, 234-242.

Hotamisligil, G.S., Shargill, N.S., Spiegelman, B.M., 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259, 87-91.

Ishida, T., 2007. Wellness care and weight management for dog-investigation of body fat ratio in dogs. Infovets 116, 34-36.

Ishioka, K., Omachi A., Sagawa M., Shibata H., Honjoh T., Kimura K., Saito M., 2006. Canine adiponectin: cDNA structure, mRNA expression in adipose tissues and reduced plasma levels in obesity. Research in Veterinary Science 80, 127-132.

Jeong, Y.J., Bong, J.G., Park, S.H., Choi, J.H., Oh, H.K., 2011. Expression of leptin, leptin receptor, adiponectin, and adiponectin receptor in ductal carcinoma in situ and incasive breast cancer. Journal of Breast Cancer 14, 96-103.

Jeusette, I., Greco, D., Aquino, F., Detilleux, J., Peterson, M., Romano, V., Torre, C., 2010. Effect of breed on body composition and comparison between various methods to estimate body composition in dogs. Research in Veterinary Science 88, 227-232.

Kadowaki, T., Yamauchi, T., Kubota, N., Hara, K., Ueki, K., Tobe, K., 2006. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. The Journal of Clinical Investigation 116, 1784-1792.

Kanno, T., Maekawa, M., 2004. Influence of fibrate treatment on malondialdehyde-modified LDL concentration. Clinica Chimica Acta 339, 97-103.

Kawasumi, K., Suzuki, T., Fujiwara, M., Mori, N Yamamoto, I., Arai, T., 2012. New Criteria of Hyperlipidemia with Insulin Resistance in Dogs. Journal of Animal and Veterinary Advances 11, 3950-3952.

Kealy, R.D., Lawler, D.F., Ballam, J.M., Mantz, S.L., Biery, D.N., Greeley, E.H., Lust, G., Segre, M., Smith, G.K., Stowe, H.D., 2002. Effects of diet restriction on life span and age-related changes in dogs. Journal of American Veterinary Medicine Association. 220, 1315-1320.

Kerouz, N.J., Horsch, D., Pons, S., Kahn, C.R., 1997. Differential regulation of insulin receptor substrates-1 and -2(IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (ob/ob) mouse. The

journal of clinical investigation 100, 3164-3172.

Kershaw, E.E., Flier, J.S., 2004. Adipose tissue as an endocrine organ. The Journal of clinical endocrinology and metabolism 89, 2548-2556.

Kim, S.P., Ellmerer, M., Van Citters, G.W., Bergman, R.N., 2003. Primacy of hepatic Insulin resistance in the development of the metabolic syndrome induced by an isocaloric moderate-fat diet in the dog. Diabetes 52, 2453-2460.

Kohsaka, A., Laposky, A.D., Ramsey, K.M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F.W., Bass, J., 2007. High-fat diet disrupts behavioral and molecular circadian rhythms in mice. Cell Metabolism, 6, 414-442.

Kollias, A., Tsiotra, P.C., Ikonomidis, I., Maratou, E., Mitrou, P., Kyriazi, E., Boutati, E., Lekakis, J., Economopoulos, T., Kremastinos, D.T., Dimitriadis, G., Raptis, S.A., 2011. Adiponectin levels and expression of adiponectin receptors in isolated monocytes from overweight patients with coronary artery disease. Cardiovascular diabetology10, 14-26.

Kondo, A., Manabe, M., Saito, K., Maekawa, M., Kanno, T., 2002. Insulin treatment prevents LDL oxidation in patients with diabetes. Journal of atherosclerosis and thrombosis 9, 280-287.

Lawler, D.F., Larson, B.T., Ballam, J.M., Smith, G.K., Biery, D.N., Evans, R.H., Greeley, E.H., Segre, M., Stowe, H.D., Kealy, R.D., 2008. Diet restriction and ageing in the dog: major observations over two decades. The British Journal of Nutrition 99, 793-805.

Lee, P., Mori, A., Coradini, M., Mori, N., Sagara, F., Yamamoto, I., Rand, J.S., Arai, T., 2013. Potential predictive biomarkers of obesity in Burmese cats. Veterinary Journal 195, 221-227.

Lee, P., Mori, A., Takemitsu, H., Yamamoto, I., Arai, T., 2011. Lipogenic gene expression in abdominal adipose and liver tissues of diet-induced overweight cats. Veterinary Journal 190, 150-153.

Lee, R.K., Hittel, D.S., Nyamandi, V.Z., Kang, L., Soh, J., Sensen, C.W., Shearer, J., 2012. Unconventional microarray design reveals the response to obesity is largely tissue specific: analysis of common and divergent responses to diet-induced obesity in insulin-sensitive tissues. Applied Physiology, Nutrition, and Metabolism 37, 257-268.

Leth, H., Andersen, K.K., Frystyk, J., Tarnow, L., Rossing, P., Parving, H.H., Flyvbjerg, A., 2008. Elevated levels of high-molecular-weight adiponectin in

Type 1 diabetes. Journal of Clinical Endocrinology and Metabolism 93, 3186-3191.

Li, G., Lee, P., Yamamoto, I., Mori, N., Arai, T., 2013. Pilot study to determine peripheral blood leukocyte mRNA expression profile of energy homeostasis genes to identify potential predictive biomarkers of obesity in dogs. Journal of Animal and Veterinary Advances 12, 69-75.

Liew, C.C., Ma, J., Tang, H.C., Zheng, R., Dempsey, A.A., 2006. The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool. The Journal of Laboratory and Clinical Medicine 147, 126-132.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2–ΔΔCT method. Methods 25, 402-408.

Lund, E.M., Armstrong, P.J., Kirk, C.A., Klausner, J.S., 2006. Prevalence and risk factors for obesity in adult dogs from private US veterinary practices. International Journal of Applied Research in Veterinary Medicine 4, 177-186.

Ma Y., Liu D., 2013. Hydrodynamic delivery of adiponectin and adiponectin receptor 2 gene blocks high-fat diet-induced obesity and insulin resistance. Gene Therapy 20, 846-852.

Magori, E., Nakamura, M., Inoue, A., Tanaka, A., Sasaki, N., Fukuda, H., Mizutani, H., Sako, T., Kimura, N., Arai, T., 2005. Malate dehydrogenase activities are lower in some types of peripheral leucocytes of dogs and cats with Type 1 Diabetes Mellitus. Research in Veterinary Science 78, 39-44.

Markwell, P.J., Van Erk, W., Parkin, G. D., Sloth C. J., Shantz-Christienson, T., 1990. Obesity in the dog. Journal of Small Animal Practice 31, 533-537.

Matsunami, T., Sato, Y., Ariga, S., Sato, T., Shimomura, T., Kashimura, H., Hasegawa, Y., Yukawa, M., 2011. Regulation of synthesis and oxidation of fatty acids by adiponectin receptors (AdipoR1/R2) and insulin receptor substrate isoforms (IRS-1/-2) of the liver in a non alcoholic steatohepatitis animal model. Metabolism 60, 805-814.

Mawby, D.I., Bartges, J.W., d'Avignon, A., Laflamme, D.P., Moyers, T.D., Cottrell, T., 2004. Comparison of various methods for estimating body fat in dogs. Journal of American Animal Hospital Association 40, 109-114.

Mendoza, J., Pévet, P., Challet, E., 2008. High-fat feeding alters the clock synchronization to light. The Journal of Physiology 586, 5901-5910.

Mori, A., Lee, P., Takemitsu, H., Iwasaki, E., Kimura, N., Yagishita, M., Hayasaka, M., Arai, T., 2009. Decreased gene expression of insulin signaling genes in insulin sensitive tissues of obese cats. Veterinary Research Communications 33, 315-329.

Mori, N., Lee, P., Kondo, K., Kido, T., Saito, T., Arai, T., 2011. Potential use of cholesterol lipoprotein profile to confirm obesity status in dogs. Veterinary Research Communication 35, 223-235.

Mori, N., Lee, P., Muranaka, S., Sagara, F., Takemitsu, H., Nishiyama, Y., Yamamoto, I., Yagishita, M., Arai, T., 2010. Predisposition for primary hyperlipidemia in Miniature Schnauzers and Shetland sheepdogs as compared to other canine breeds. Research in Veterinary Science 88, 394-399.

Mori, N., Lee, P., Yamamoto, I., Arai, T., 2012. Elevated plasma adiponectin level and peripheral blood leukocyte adiponectin receptor expression in dogs suffering from insulin defciency. Open Veterinary Science Journal 6:1-7.

Mori, N., Sakai, M., Yamamoto, I., Arai, T., 2011. Alternation of physical indexes and plasma biochemical makers in overweight dogs induced by high-fat diet feeding. Research Journal of Veterinary Science 4:14-19.

Mori, N., Kawasumi, K., Suzuki, T., Yamamoto, I., Kobayashi, M., Arai, T. 2012. Establishment of temporary criteria for metabolic syndrome (MS) diagnosis and assessment of the occurrence rate of MS in cats. Journal of Animal and Veterinary Advances 11, 615-617.

Moussa, S.A., 2008. Oxidative stress in diabetes mellitus. Romanian Journal of Biophysics 18, 225-236.

Nadler, S.T., Stoehr, J.P., Schueler, K.L., Tanimoto, G., Yandell, B.S., Attie, A.D., 2000. The expression of adipogenic genes is decreased in obesity and diabetes mellitus. Proceedings of the National Academy of Sciences of the United States of America 97, 11371-11376.

Nguyen, P.G., Dumon, H.J., Siliart, B.S., Martin, L.J., Sergheraert, R., Biourge, V.C., 2004. Effects of dietary fat and energy on body weight and composition after gonadectomy in cats. American Journal of Veterinary Research 65, 1708-1713.

Osto, M., Zini, E., Reusch, C.E., Lutz, T.A., 2013. Diabetes from humans to cats. General and Comparative Endocrinology 182, 48-53.

Park, J., Choe, S.S., Choi, A.H., Kim, K.H., Yoon, M.J., Suganami, T., Ogawa, Y., Kim, J.B., 2006. Increase in glucose-6-phosphate dehydrogenase in adipocytes

stimulates oxidative stress and inflammatory signals. Diabetes 55, 2939-2949.

Park, J., Rho, H.K., Kim, K.H., Choe, S.S., Lee, Y.S., Kim, J.B., 2005. Overexpression of glucose-6-phosphate dehydrogenase is associated with lipid dysregulation and insulin resistance in obesity. Molecular and Cellular Biology 25, 5146-557.

People's Dispensary for Sick Animals. 50 Percent of UK dogs could die early due to obesity epidemic warns charity. Available from: http://www.pdsa.org.uk/about-us/media-pr-centre/news/1182. Accessed July 16, 2012.

Pischon, T., Girman, C.J., Rifai, N., 2005. Association between dietary factors and plasma adiponectin concentration in men. The American Journal of Clinical Nutrition 81, 780-786.

Rand, J.S., Fleeman, L.M., Harrow, H.A., Appleton, D.J., Lederer, R., 2004. Canine and feline diabetes mellitus: nature or nuture? The Journal of nutrition 134, 2072-2080.

Rondinone, C.M., Wang, L.M., Lonnroth, P., Wesslau, C., Pierce, J.H., Smith, U., 1997. Insulin receptor substrate (IRS) 1 is reduced and IRS-2 is the main docking protein for phosphatidylinositol 3-kinase in adipocytes from subjects with non-insulin-dependent diabetes mellitus. Proceeding of the National Academy of the Sciences of the United States of America 94, 4171-4175.

Scarlett, J.M., Donoghue, S., Saidla, J., Wills, J., 1994. Overweight cats: prevalence and risk factors. International Journal of Obesity and Related Metabolic Disorders 1:S22-8.

Serpillon, S., Floyd, B.C., Gupte, R.S., George, S., Kozicky, M., Neito, V., Recchia, F., Stanley, W., Wolin, M.S., Gupte, S.A., 2009. Superoxide production by NAD(P)H oxidase and mitochondria is increased in genetically obese and hyperglycemic rat heart and aorta before the development of cardiac dysfunction. The role of glucose-6- phosphate dehydrogenase-derived NADPH. American Journal of Physiology 297, 153-162.

Shen, Y.Y., Charlesworth, J.A., Kelly, J.J., Loi, K.W., Peake, P.W., 2007. Up-regulation of adiponectin, its isoforms and receptors in end-stage kidney disease. Nephology, Dialysis, Transplantation 22, 171-178.

Sherman, H., Genzer, Y., Cohen, R., Chapnik, N., Madar, Z., Froy, O., 2012. Timed high-fat diet resets circadian metabolism and prevents obesity. FASEB Jounal 26, 3493-3502.

Silva, F.M., de Almeida, J.C., Feoli, A.M., 2011. Effect of diet on adiponectin levels in blood. Nutrition Reviews 69, 599-612.

Staiger, H., Kaltenbach, S., Staiger, K., Stefan, N., Fritsche, A., Guirguis, A., Peterfi, C., Weisser, M., Machicao, F., Stumvoll, M., Haring, H.U., 2004. Expression of adiponectin receptor mRNA in human skeletal muscle cells is related to in vivo parameters of glucose and lipid metabolism. Diabetes 53, 2195-2201.

Stefanovski, D., Richey, J.M., Woolcott, O., Lottati, M., Zheng, D., Harrison, L.N., Ionut, V., Kim, S.P., Hsu, I., Bergman, R.N., 2011. Consistency of the disposition index in the face of diet induced insulin resistance: potential role of FFA. PLoS One 6, e18134.

Stone, R., Berghoff, N., Steiner, J.M., Zoran, D., 2009. Use of a bioelectric impedance device in obese and lean healthy dogs to estimate body fat percentage. Veterinary Therapeutics 10, 59-70.

Sun, Y., Liu, S., Ferguson, S., Wang, L., Klepcyk, P., Yun, J.S., Friedman, J.E., 2002. Phosphoenolpyruvate carboxykinase overexpression selectively attenuates insulin signaling and hepatic insulin sensitivity in transgenic mice. The Journal of Biological Chemistry 277, 23301-23307.

Tan, G.D., Debard, C., Funahashi, T., Humphreys, S.M., Matsuzawa, Y., Frayn, K.N., Karpe, F., Vidal, H., 2005. Changes in adiponectin receptor expression in muscle and adipose tissue of type 2 diabetic patients during rosiglitazone therapy. Diabetologia 48, 1585-1589.

Tanaka, A., Inoue, A., Takeguchi, A., Washizu, T., Bonkobara, M., Arai, T., 2005. Comparison of expression of glucokinase gene and activities of enzymes related to glucose metabolism in livers between dog and cat. Veterinary Research Communication 29, 477-485.

Takeguchi, A., Urabe, S., Tanaka, A., Sako, T., Washizu, T., Morinaga, N., Kimura, N. and Arai, T., 2005. Activities of enzymes in some types of peripheral leucocytes may reflect the differences in nutrient metabolism between dogs and cats. Research in Veterinary Science 78, 21-24.

Tamura, A., Watanabe, T., Nasu, M., 2003. Effects of atorvastatin and pravastatin on malondialdehyde-modified LDL in hypercholesterolemic patients. Circulation Journal 10, 816-20.

Terpstra, A.H., Sanchez-Muniz, F.J., West, C. E., Woodward, C.J., 1982. The density profile and cholesterol concentration of serum lipoproteins in domestic and

laboratory animals. Comparative biochemistry and physiology B, Comparative Physiology 71, 669-673.

Tsuchida, A., Yamauchi, T., Ito, Y., Hada,Y., Maki, T., Takekawa, S., Kamon, J., Kobayashi, M., Suzuki, R., Hara, K., Kubota, N., Terauchi, Y., Froguel, P., Nakae, J., Kasuga, M., Accili, D., Tobe, K., Ueki, K., Nagai, R., Kadowaki, T., 2004. Insulin/Foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity. Journal of Biological Chemistry 279, 30817-30822.

Tvarijonaviciute, A., Ceron, J.J., Holden, S.L., Cuthbertson, D.J., Biourge, V., Morris, P.J., German, A.J., 2012. Obesity-related metabolic dysfunction in dogs: a comparison with human metabolic syndrome. BMC Veterinary Research 8, 147.

Umeda, T., Bauer, J.E., Otsuji, K., 2006. Weight loss effect of dietary diacylglycerol in obese dogs. Journal of Animal Physiology and Animal Nutrition 90, 208-215.

Verkest, K.R., Bjornvad, C.R., 2012. Understanding adiponectin in dogs and cats: a work in progress. Veterinary Journal 193, 4-5.

Verkest, K.R., Fleeman, L.M., Morton, J.M., Ishioka, K., Rand, J.S., 2011. Compensation for obesity-induced insulin resistance in dogs: assessment of the effects of leptin, adiponectin, and glucagon-like peptide-1 using path analysis. Domestic Animal Endocrinology 41, 24-34.

Verkest, K.R., Rand, J.S., Fleeman, L.M., Morton, J.M., Richards, A.A., Rose, F.J., Whitehead, J.P., 2011. Distinct adiponectin profles might contribute to differences in susceptibility to type 2 diabetes in dogs and humans. Domestic Animal Endocrinology 41, 67-73.

Verkest, K.R., Rose, F.J., Fleeman, L.M., Rand, J.S., Morton, J.M., Richards, A.A., Ishioka, K., Whitehead, J.P., 2011. Adiposity and adiponectin in dogs: investigation of causes of discrepant results between two studies. Domestic Animal Endocrinology 41, 35-41.

Visvikis-Siest, S., Marteau, J.B., Samara, A., Berrahmoune, H., Marie, B., Pfister, M., 2007. Peripheral blood mononuclear cells (PBMCs): a possible model for studying cardiovascular biology systems. Clinical Chemistry Laboratory Medicine 45, 1154-1168.

Wakshlag, J.J., Struble, A.M., Levine, C.B., Bushey, J.J., Laflamme, D.P., Long, G.M., Wang, Y., Seburn, K., Bechtel, L., Lee, B.Y., Szatkiewicz, J.P., Nishina, P.M.,

Naggert, J.K., 2011. The effects of weight loss on adipokines and markers of inflammation in dogs. The British journal of Nutrition1, 11-14.

Wang, F., Zhao, Y., Niu, Y., Wang, C., Wang, M., Li, Y., Sun, C., 2012. Activated glucose-6-phosphate dehydrogenase is associated with insulin resistance by upregulating pentose and pentosidine in diet-induced obesity of rats. Hormone and Metabolism Research 44, 938-942.

Washizu, T., Tanaka, A., Sako, T., Washizu, M., Arai, T., 1999. Comparison of the activities of enzymes related to glycolysis and gluconeogenesis in the liver of dogs and cats. Research in Veterinary Science 67, 205-206.

White, G.A., Hobson-West, P., Cobb, K., Craigon, J., Hammond, R., Millar, K.M., 2011. Canine obesity: is there a difference between veterinarian and owner perception? The Journal of Small Animal Practice 52, 622-626.

White, M.F., 1998. The IRS signaling system: a network of docking proteins that mediate insulin and cytokine action. Recent progress in hormone research 53, 119-138.

Whitney, M.S., 1992. Evaluation of hyperlipidemias in dogs and cats. Seminars in veterinary medicine and surgery (Small Animal) 7, 292-300.

Withers, D.J., Gutierrez, J.S., Towery, H., Burks, D.J., Ren, J.M., Previs, S., Zhang Y., Bernal, D., Pons, S., Shulman, G.I., Bonner-Weir, S., White, M.F. 1998. Disruption of IRS-2 causes type 2 diabetes in mice. Nature. 391, 900-904.

Xenoulis P.G., Cammarata P.J., Walzem R.L., Macfarlane R.D., Suchodolski J.S., Steiner J.M., 2013. Novel lipoprotein density profiling in healthy dogs of various breeds, healthy miniature schnauzers, and miniature schnauzers with hyperlipidemia. BMC Veterinary Research; 9:47.

Xenoulis, P.G., Steiner J.M., 2010. Lipid metabolism and hyperlipidemia in dogs. Veterinary Journal 183: 12-21.

Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., Murakami, K., Ohteki, T., Uchida, S., Takekawa, S., Waki, H., Tsuno, NH., Shibata, Y., Terauchi, Y., Froguel, P., Tobe, K., Koyasu, S., Taira, K., Kitamura, T., Shimizu, T., Nagai, R., Kadowaki, T., 2003. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423, 762-769.

Yang, Z.H., Miyahara, H., Takeo, J., Katayama, M., 2012. Diet high in fat and sucrose induces rapid onset of obesity-related metabolic syndrome partly through rapid

response of genes involved in lipogenesis, insulin signalling and inflammation in mice. Diabetology and Metabolic Syndrome. 4, 32.

Zoran, D.L., 2010. Obesity in dogs and cats: a metabolic and endocrine disorder. The Veterinary Clinics of North America. Small Animal Practice 40, 221-239.

## Acknowledgements

This thesis would not have been possible without the contributions off several people. Too all of these people, I wish to express my gratitude.

First and foremost, grateful acknowledgement is made to my supervisor-Professor Toshiro Arai, whose encouragement and unwavering support has sustained me through frustration and depression throughout my Ph.D studies. His tremendous assistance in having gone through the draft versions of all the papers and this thesis several times as well as his great care in life deserves more thanks than I can find words to express.

I am also greatly indebted to Dr. Ichiro Yamamoto, who gave me considerable help by means of patient guidance, generous assistance and invaluable advice throughout my experimental research period in Japan. And I would like to thank all the colleagues for whom I have great regard, post-doctor Nobuko Mori, Megumi Fujiwara and Dongwei Zhao; Dr. Hiroshi Takemitus; Ph.D. student Shingo Ishikawa, and other students in Biochemistry lab. And I wish to extend my sincere thanks to Dr. Peter Lee, for proofreading and correcting the English of all my manuscripts and sharing his knowledge with me, throughout my doctoral course.

I would like to thank Dr. Kawasumi and Togashira Animal Hospital, Celebre Animal Hospital, Lion Animal Hospital, Sawamura Animal Hospital, Grace Animal Hospital, Sakura Animal Hospital, for providing assistance and collecting the experimental animal blood samples.

In addition, I would like to offer special thanks to Nippon Medical School

Foundation and Japan Student Services Organization, which offered me the scholarship and financial support for my Ph.D. studies in Japan.

Last my thanks would go to my beloved family and friends for their boundless love and whole-hearted support over all these past years. Without their support, the completion of my Ph.D. would not have been possible.