The Role of Adipose Tissue in

Canine Cranial Cruciate Ligament Disease

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By

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List of Abbreviations

ACL	Anterior cruciate ligament
Acrp30	Adipocyte complement-related protein of 30 kDa
AG	Alex German
AGC	Aggrecan
AI	Asymmetry index
apM1	Adipose most abundant gene transcript 1
BAT	Brown adipose tissue
BCS	Body condition score
BMI	Body mass index
CCL	Cranial cruciate ligament
CCLD	Cranial cruciate ligament disease
COL-1	Type 1 collagen, alpha two chain (COL1A2)
DEPC	Diethylpyrocarbonate
DEXA	Dual-energy X-Ray absorptiometry
DMMB	1,9-dimethylmethylene blue
DMSO	Dimethyl sulfoxide
EC	Eithne Comerford
ECVS	European College of Veterinary Surgeon
ELISA	Enzyme-linked immunosorbent assay
FS	Falling slope
Fx	Mediolateral force
Fy	Craniocaudal force
Fz	Vertical force
GBP28	Gelatin binding protein of 28 kDa
GRF	Ground reaction force
IL	Interleukin
IPFP	Infrapatellar fat pad
kg	Kilogram

m	Metre
MMP	Matrix metalloproteinase
MCP-1	Macrophage chemotactic protein-1
mt	Month
MVM	Master of Veterinary Medicine
n	Number
OA	Osteoarthritis
PBEF	Pre-B-cell colony-enhancing factor
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PVF	Peak vertical force
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RA	Rheumatoid arthritis
RCVS	Royal College of Veterinary Surgeon
RPS-13	Ribosomal protein S13
RS	Rising slope
SC	Subcutaneous
SD	Standard deviation
sGAG	Glycosaminoglycan
TNF-α	Tumor necrosis factor-α
TPLO	Tibial plateau levelling osteotomy
TTA	Tibial tuberosity advancement
TTO	Triple tibial osteotomy
VI	Vertical impulse
WAT	White adipose tissue
WS	Wipawee Saengsoi

Acknowledgements

Abstract

The Role of Adipose Tissue in Canine Cranial Cruciate Ligament Disease

Wipawee Saengsoi

Cranial cruciate ligament disease (CCLD) is one of the most important causes of pelvic limb lameness in dogs, and its prevalence is increased in dogs that are overweight. To date, the mechanisms underlying the association between obesity and CCLD in dogs have not been clarified. Therefore, the general aims of this thesis were to examine the role of both the mechanical and humoral effects of adipose tissue on the clinical presentation and pathogenesis of dogs with CCLD. Dogs suffering from CCLD were clinically evaluated by calculating lameness scores, force plate analysis, radiographic examination, and dual-energy X-ray absorptiometry (DEXA). Increased bodyweight was associated with severity of lameness, as determined by force plate analysis, but there was no association with body fat mass determined by DEXA. In vitro studies were also used to examine interactions between adipose tissue and the canine cruciate ligament. In co-culture experiments, adipose tissue had a catabolic effect on glycosaminoglycan content in CCL explants. Furthermore, various associations between key adipokines (adiponectin, leptin, visfatin) with disease biomarkers in CCL tissues and joint fluid were discovered. For example, an association was identified between synovial fluid TNF-α concentration and the gene expression of adiponectin and visfatin in infrapatellar fat pad (IPFP) adiponectin and subcutaneous (SC) fat, respectively. Associations were also seen between IPFP leptin gene expression and MMP-13 gene expression, and between both IPFP and SC visfatin gene expression and CCL TNF-α gene expression. Finally, synovial fluid leptin concentration was positively associated with the degree of lameness. In conclusion, the studies undertaken in this thesis have demonstrated that adipose tissue may have both mechanical and humoral effects on the cruciate ligament in dogs. These findings provide a basis for further studies into the pathogenesis of CCLD, as well as revealing possible targets for therapeutic intervention.

Chapter 1 Introduction

1.1 Obesity in man and companion animals

Adiposity is fat stored in the fatty tissue of the body. Obesity is defined as a disease where excess body fat has accumulated and frequently results in various adverse effects on health and longevity (National Institutes of Health, 1985). Increased body fat accumulation is associated with an increase in adipocyte size and, in extreme obesity, adipocyte numbers are also increased (Hirsch and Batchelor, 1976). Usually, it requires a prolonged period of imbalance between energy intake and expenditure, with intake calories exceeding calories burned, in order for obesity to develop (National Institutes of Health, 1985).

1.1.1 Assessment of obesity

There are different methods that can be used to assess body condition in man, for example body mass index (BMI), anthropometrics, skinfold thickness, bioimpedance, dilution technique, hydrostatic weighing, air displacement plethysmography, dual-energy X-ray absorptiometry and computerised tomography scans (Beechy et al., 2012). Most epidemiological studies and clinical practices commonly use BMI given its strong correlation with fat mass densitometry and due to the fact that neither specialist nor extra equipment is required (Kopelman, 2000). BMI can be calculated by dividing body weight (kg) by height (m^2) (VanItallie et al., 1990). Using the classification from the WHO, human beings can be classified as underweight (<18.5 kg/m^2), normal (18.5-24.9 kg/m^2), overweight (25.0-29.9 kg/m^2), obese class I (30.0-34.9 kg/m^2), obese class II (35.0-39.9 kg/m^2) and obese class III (≥40.0 kg/m^2). This is generally used for adults of 20 years and older (WHO, 2000). However, the associations of BMI and comorbidities are not always stable in different populations (Swinburn et al., 1996). BMI does not distinguish between the type of fat such as subcutaneous (SC) and visceral fat. Therefore, other assessment methods

such as waist circumference and waist-to-hip ratio should be used to measure intra-abdominal (visceral) fat, which has been shown to be more connected to obesity-related illness (Han et al., 1995).

Bodyweight measurement is routinely used in veterinary practice, and it can be the first indicator for observing weight loss or gain in companion animals (German, 2006). Companion animals are classified as overweight when their bodyweight is more than 10% above their ideal weight and classified as obese when their bodyweight exceeds 20% of their optimums (German, 2006). However, although bodyweight provides an objective measurement of body mass, it cannot indicate adiposity or body composition (Toda et al., 1998). Several methods have been developed to assess obesity in dogs and cats (Table 1.1). Body condition scoring (BCS) is often used by veterinarians because it is practical, inexpensive and non-invasive to the animal providing a semi-quantitative method of evaluating body composition (Burkholder, 2000). BCS uses visual assessment and palpation on specific body regions including rib cage, waist, lumbar region, and base of the tail. Although various systems exist (Scarlett and Donoghue, 1998, German et al., 2006, Witzel et al., 2014), it is now recommended that the 9-point BCS system (Laflamme, 1997a, Laflamme, 1997b) be used exclusively to aid consistency (Freeman et al., 2011). It has been validated for repeatability, reproducibility by trained investigators and owners, as well as correlating well with body fat composition in dogs and cats (German et al., 2006). In this scoring system, a score of 4 or 5 represents an ideal body condition, a score of 1 represents an emaciated animal and a score of 9 represents a grossly obese animal (Laflamme, 1997a, Laflamme, 1997b). BCS also can determine the approximate degree of excess bodyweight in obese dogs with each score correlating with approximately 10% to 15% of excess bodyweight (German et al., 2009).

Table 1.1 Methods for body composition analysis in dogs and cats.

(adapted from German et al., 2006)

Common research techniques	Common clinical methods
Chemical analysis	Bodyweight
Densitometry	Morphometric methods
Total body water	Body condition score
Total body potassium	9-Point scale
Absorptiometry	5-Point scale
Photon absorptiometry	
Single-photon absorptiometry	
Dual-photon absorptiometry	**Other methods**
X-ray absorptiometry	Muscle metabolite markers
Single-energy X-ray absorptiometry	Neutron activation analysis
Dual-energy X-ray absorptiometry	Electrical conductance
Ultrasound	Near infrared interactance
Electrical impedance	Computed tomography
	Magnetic resonance imaging

Dual-energy X-ray absorptiometry (DEXA) is a non-invasive technique that can be used to quantify bone density and body composition (including lean tissue mass, fat mass, and bone mineral content) in humans (Lang et al., 2015, Rae et al., 2016) and animals (Lang et al., 2015, Rae et al., 2016). The method by which DEXA estimates body composition is based on the principle that the intensity of X-ray beams is attenuated in proportion to tissue mass they pass through. The X-ray beam, which is composed of two different frequencies, is impeded by bone, fat and lean tissue (Grier et al., 1996). Bone mineral content, lean body mass, and fat are then calculated with commercially available software and compared with standard values in an object of known density (Grier et al., 1996). The advantages of DEXA include a short scan time, precise measurement with its high-resolution images, the ability to analyse different body regions, low radiation dose and being relatively inexpensive (Blake and Fogelman, 2007). DEXA is sensitive enough to measure relatively small changes in bone mass, so it has been used for screening and diagnosis of metabolic conditions associated with bone loss such as osteoporosis, measuring fracture healing, growth studies and sports medicine in man (Maffulli et al., 1997, Kesman et al., 2010, Lerch et al., 2013). In addition, measurement of total body composition by DEXA is useful in nutritional-related studies for determining energy expenditure and stores, protein mass, and for defining relative hydration (Grier et al., 1996). In animals, DEXA is used in the study of overweight animals as well as in neonatal and pharmacological studies (Grier et al., 1996, German et al., 2009). Furthermore, recent studies have effectively used DEXA to monitor canine body composition during weight loss (Larsson et al., 2014, German et al., 2015).

1.1.2 Epidemiology of obesity

In 2013, one third of the world's adult population were overweight or obese (Ng et al., 2014). With time there is an increasing risk of death due to obesity due to fatal co-morbidities such as coronary heart disease, hypertension, stroke, certain types of cancer and type 2 diabetes mellitus (Haslam and James, 2005). In England,

62% of adults and 28% of children aged between 2 and 15 are overweight or obese (GOV.UK, 2015). Being overweight and obese also has financial implications for the wider economy: the costs for the National Health Service (England) to treat overweight, obesity and related co-morbidities ranged between £479.3 million in 1998 (National Audit Office, 2001) and £4.2 billion in 2007 (Butland et al., 2007). In the United States, obesity related costs are predicted to reach as much as $956 billion by 2030 (Wang et al., 2008). Once considered only to be a problem of high income countries, obesity is now also on the rise in low- and middle-income countries across many demographic groups, especially in urban areas (Mitchell and Shaw, 2015, Atay and Bereket, 2016).

As in men, the incidence of obesity in companion animals is also increasing. Obesity is the most common medical disorder that affects health of dogs and cats, especially in developed countries (Clark and Hoenig, 2016), with recent reports estimating that 34% to 59% of dogs (McGreevy et al., 2005, Courcier et al., 2010b) and 26% to 63% of cats (Colliard et al., 2009, Courcier et al., 2010a, Cave et al., 2012) are overweight or obese.

1.1.3 Causes of obesity

Factors influencing the development of overweight and obesity in humans include diet, physical activity patterns, environment, society and individual susceptibility (WHO, 2000). Like in humans, the causes of obesity in pets are multifactorial. Age, genetics (or breed), social and environmental conditions (dietary exposure and physical activity) play a major role in the development of the disease (Edney and Smith, 1986, Heber, 2010, Vanden Bosch et al., 2014). In addition, risk factors for dog and cat obesity also include sex, gonadal status and neutering (Root et al., 1996, Fettman et al., 1997), hormonal influences such as hypothyroidism; (Jeusette et al., 2006), some infectious diseases such as canine distemper virus; (Verlaeten et al., 2001), rapid early life weight gain, behavioural factors and owner factors (German, 2016a). However, a positive mismatch between energy intake and

expenditure is the key factor of development of obesity and overfeeding in dogs and cats appear to be the main cause of the disease (German, 2006).

1.1.4 The clinical importance of obesity

Obesity is associated with various morbidities (Table 1.2) and increases mortality risk in both humans and animals (German et al., 2010b, Linder and Mueller, 2014). In man, obesity is known to increase the incidence of serious diseases such as cardiovascular disease, stroke and type 2 diabetes mellitus (Dankner et al., 2009, Friedemann et al., 2012). Obesity can also lead to neurological, pulmonary, gastrointestinal, renal and musculoskeletal disorders as well as various forms of psychopathy and a reduced quality of life (Ebbeling et al., 2002). In companion animals, obesity increases the risk of developing several health abnormalities. These include: cardiorespiratory disease, endocrine disorders, metabolic diseases, diabetes mellitus, hepatic lipidosis, urinary tract disease, dermatopathies, cancer and orthopaedic diseases (DeFronzo and Ferrannini, 1991, Scarlett and Donoghue, 1998, Clark and Hoenig, 2016).

Table 1.2 Diseases associated with obesity in man and companion animals.

(adapted from German et al., 2010, Linder and Mueller, 2014)

Body System	Species		
	Human	**Dog**	**Cat**
Endocrine and lipid metabolism	Type 2 diabetes mellitus Metabolic syndrome Dyslipidaemias	Hypothyroidism Hyperadrenocorticism Diabetes mellitus Insulin resistance Hyperinsulinaemia Metabolic syndrome	Impaired glucose tolerance Diabetes mellitus Hepatic lipidosis Dyslipidaemia Hypercholesterolemia Hypertriglyceridemia
Cardiorespiratory	Coronary heart disease Atherosclerosis Hypertension Obstructive Sleep apnoea Asthma	Cardiac alterations Tracheal collapse Airway dysfunction Hypertension Portal vein thrombosis Myocardial hypoxia Heatstroke	Cardiac alterations
Orthopaedic and impaired mobility	Osteoarthritis Musculoskeletal pain Gout	Osteoarthritis Cruciate ligament disease Humeral condylar fractures Intervertebral disc disease Hip dysplasia	Increased lameness
Neoplasia	Breast Renal Endometrial Prostatic Oesophageal Colorectal Hepatocellular	Transitional cell carcinoma Mammary carcinoma	Increased neoplasia risk
Urogenital	(Diabetic) nephropathy	Urinary tract disease Calcium oxalate urolithiasis Transitional cell carcinoma Glomerular disease Dystocia	Increased risk of urinary tract disease Urolithiasis
Alimentary	Pancreatitis Hepatic steatosis Cirrhosis	Pancreatitis Oral disease	Increased oral and gastrointestinal disease

1.1.5 Treatment and management of obesity

The key treatments of obesity in people include lifestyle management, pharmacological and surgical therapies or a combination of these methods (Quante et al., 2015). The treatment chosen depends on disease severity, the presence of weight-related complications and risks for each patient. A decreased caloric intake and increased physical activity are key lifestyle and behavioural interventions, which are considered first-line therapy for those overweight and obese (Cefalu et al., 2015). The variety of dietary options, social networks and structured weight loss programmes are increasing, helping to reduce morbidity and improve the life quality of obese people. Several drugs have been developed and approved to use for long-term weight management in humans such as the lipase inhibitor, the serotonin 2c receptor agonist and the glucagon-like peptide-1 receptor agonist (Apovian et al., 2015). However, medical or non-surgical treatment may not produce effective long-term maintenance of weight loss for the morbidly obese and therefore surgical management (bariatric surgery) may be the most feasible treatment in these cases (Quante et al., 2015). There are many techniques for bariatric surgeries including jejunoileal bypass, biliopancreatic diversion, gastroplasty and Roux-en-Y gastric bypass (Fobi, 2004). Nevertheless, these surgeries are complicated and patients undergoing these procedures need lifelong nutritional supplements and frequent medical monitoring (Fobi, 2004).

Weight management principles in companion animals are similar to these in humans. Dietary energy restriction using purpose-formulated foods are most widely used with the aim of delivering essential nutrients while reducing calorie intake (Hoenig and Rand, 2006). Moreover, there are several methods for reducing calorie density in animal foods such as reducing fat, adding moisture, or adding fiber (Laflamme, 2005). Previous study has suggested that approximately 1% of body weight loss per week prevents the risk of nutrient deficiency, loss of lean body mass and rebound weight gain (Laflamme and Hannah, 2005). Increasing physical activities

such as walking, swimming or using interactive toys are also beneficial for pets to maintain their weight loss (Wakshlag et al., 2012).

In addition to dedicated weight loss diets, feeding management and exercise, owner education is critically important for long-term successful weight control in companion animals (Bland et al., 2009). However, many owners do not recognise that their companion animals are overweight or obese. Therefore, veterinarians have an important role to evaluate the BCS of pets and discuss with clients the importance of maintaining an ideal BCS (Linder and Mueller, 2014). Weight loss drugs such as dirlotapide and mitratapide have been used in pets (Klonoff, 2007, Dobenecker et al., 2009). However, these two licensed drugs are no longer available due to unpopular usage in clinical practice (German, 2016b).

1.2 Adipose tissue and adipokines

Adipose tissue is mainly located in the SC layer and deposited around visceral organs (Cinti, 2005). The general roles of adipose tissue are to store energy in the form of lipids, provide protective cushions of internal organs and insulate the body (Rosen and Spiegelman, 2014). The amount of adipose tissue in the body is another indicator for obesity in animals and humans (National Institutes of Health, 1985, Zoran, 2010). In mammals include human, adipose tissue can be divided into two major types: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT represents the bulk of the adipose tissue in the body (Fantuzzi, 2005). It is the site of energy storage with triacylglycerols stored in adipocytes and provides insulation (Shapiro and Zinder, 1972). In contrast, BAT contains an abundance of mitochondria for its heat-generating properties and it is present in higher quantities in the new-born mammal such as human than in the adult (Nedergaard and Cannon, 2017). In addition, brown in white (brite or beige) adipose depots, which look white in the adult but are functionally close to that of BAT in the new-born, have also been distinguished in humans and small mammals (Walden et al., 2012).

Adipose or fatty tissue is loose connective tissue primarily composed of adipocytes (adipose cells or fat cells) (Cinti, 2005). Adipose tissue has a stromal vascular fraction that contains preadipocytes, connective tissue matrix, nerve tissue, vascular endothelial cells and immune cells (Frayn et al., 2003, Fain et al., 2004, Klein-Wieringa et al., 2011). In recent years, adipose tissue has been recognised as an active endocrine organ in many species (Kershaw and Flier, 2004). Adipocytes synthesise and release an array of chemical mediators, named adipokines, examples of which include adiponectin, leptin and visfatin (Lagowska and Jeszka, 2011). Adipokines act upon autocrine, paracrine and endocrine signals which have potent effects on systemic metabolic homeostasis (Kershaw and Flier, 2004). Moreover, many biological proteins including acute phase proteins, chemokines (e.g. macrophage chemotactic protein-1; MCP-1) and cytokines (e.g. tumour necrosis factor-alpha;

TNF-α, interleukin-6; IL-6) have been found to be secreted from non-adipocyte fraction of adipose tissue (Fain et al., 2004). Activation of adipose tissue macrophages within adipose depots can lead to the development of an obesity-induced pro-inflammatory state (Odegaard and Chawla, 2008), which may predispose to various diseases such as obesity-induced insulin resistance, type 2 diabetes mellitus, inflammatory bowel disease, osteoarthritis (OA) and rheumatoid arthritis (Fantuzzi, 2005, Klein-Wieringa et al., 2011, Clark and Hoenig, 2016). Therefore, adipose tissue and adipokines are involved in a complex network coordinating a variety of biological processes in the body.

1.2.1 Leptin

Leptin, a 16-kDa polypeptide containing 167 amino acids, was the first adipokine to be discovered in 1994 and was called obese protein at that time (Zhang et al., 1994). The discovery of this adipokine demonstrated that adipose tissue was not simply an organ for energy storage but, instead, was an active endocrine organ (Kershaw and Flier, 2004). Leptin is primarily produced from adipocytes and it is secreted into blood circulation in direct proportion to adipocyte volume (Lönnqvist et al., 1997). Leptin can play a role in metabolic signalling throughout the body system (Myers et al., 2008). It often increases with increasing adiposity and is significantly correlated with total body fat mass, both in humans and companion animals (Monti et al., 2006, Ishioka et al., 2007, Ruhl et al., 2007, Martin-Gimenez et al., 2016). The circulating leptin concentration displays a diurnal rhythm and is at its highest concentrations at night, but its concentration can also be affected by meal times (Saladin et al., 1995). In addition, increased leptin expression and secretion are regulated by several factors such as insulin, glucocorticoids, TNF-α and oestrogens (Margetic et al., 2002). Conversely, its function is reduced by β3-adrenergic activity, somatostatin, growth hormone and free fatty acids (Margetic et al., 2002). Leptin plays critical roles in regulating appetite, energy expenditure and body weight homeostasis via hypothalamic pathways (Zhang et al., 1994, Elmquist, 2001). Leptin

stimulates fatty acid oxidation and glucose uptake (Minokoshi et al., 2002). It also has effects on the immune system by influencing T lymphocyte function, monocyte activation, phagocytosis, and cytokine production (Lord et al., 1998, Farooqi et al., 2002). An increased concentration of circulating leptin was found to be associated with negative outcomes in cardiovascular disease in humans (Schulze and Kratzsch, 2005) and in dogs (Fonfara et al., 2011). Leptin is also expressed in other tissues including the gastrointestinal system, skeletal muscle and the female reproductive system (Hoggard et al., 1997, Wang et al., 1998, Liu et al., 2003).

1.2.2 Adiponectin

Adiponectin was discovered in 1995 and has been described by different names including Acrp30 (adipocyte complement-related protein of 30 kDa), apM1 (adipose most abundant gene transcript 1), GBP28 (gelatin binding protein of 28 kDa) and adipoQ (Scherer et al., 1995, Hu et al., 1996, Maeda et al., 1996, Nakano et al., 1996). Adiponectin is a 30-kDa polypeptide hormone containing an N-terminal signal sequence, a variable region, a collagen-like domain and a C-terminal globular domain (Scherer et al., 1995). Adiponectin is mainly secreted from adipose tissue during adipocyte differentiation (Meier and Gressner, 2004). Its secretion is stimulated by insulin and PPARγ receptor agonists, whereas TNF-α (tumor necrosis factor-α) and PPARα receptor agonists suppress it (Rajala and Scherer, 2003). The main functions of adiponectin include enhancing insulin sensitivity, increasing fatty acid oxidation and reducing gluconeogenesis in liver and skeletal muscle (Kershaw and Flier, 2004, Galic et al., 2010). In addition, adiponectin has protective properties with regards to atherosclerosis by reducing the inflammatory reaction in atherogenesis (Pischon et al., 2004). Many studies have demonstrated that plasma adiponectin is negatively associated with body fat mass, BMI, fasting insulin and plasma triglyceride concentrations (Cnop et al., 2003, Goropashnaya et al., 2009). Decreased serum adiponectin concentration is related to an increased risk of cardiovascular disease and type 2 diabetes mellitus, whilst a high serum adiponectin concentration has been

reported in cases of renal failure, type 1 diabetes, and anorexia (Weyer et al., 2001, Schondorf et al., 2005, Lagowska and Jeszka, 2011). In dogs and cats, adiponectin is also produced by mature adipocytes and highly expressed in WAT (Zoran, 2010). The nucleotide and amino acid sequences of companion animal adiponectin show strong similarity to those of other species especially between dogs and human (Brunson et al., 2007). In dogs, plasma adiponectin concentration negatively correlates with fat mass, and the circulating concentration of adiponectin is markedly lower in obese than in normal-weight dogs (Brunson et al., 2007, Piantedosi et al., 2016). Moreover, increased body fat mass is negatively correlated with low- and high-molecular weight of plasma adiponectin ratio in cats (Bjornvad et al., 2014). These studies suggest similar roles between adiponectin and leptin in the development of metabolic conditions such as insulin resistance, and type 2 diabetes in companion animals (Zoran, 2010). However, there are still relatively few studies on the roles of adiponectin in the development of diseases in dogs and cats.

1.2.3 Visfatin

Visfatin was initially discovered in 1994 as a growth factor for the maturation of B lymphocyte precursors, so it was named pre-B-cell colony-enhancing factor (PBEF) (Samal et al., 1994). Later, it was termed nicotinamide phosphoribosyltransferase (Nampt) due to its enzymatic role in nicotinamide adenine dinucleotide (NAD) biosynthesis (Rongvaux et al., 2002). Visfatin is 52-kDa adipokine synthesised by many tissues including leucocytes of peripheral blood (Samal et al., 1994), adipose tissue macrophages (Curat et al., 2006), hepatocytes (Garten et al., 2010), skeletal muscles (Costford et al., 2010), chondrocytes (Gosset et al., 2008) and synovium (Laiguillon et al., 2014). The connection of serum visfatin concentration and obesity status is controversial as well as its expression in different adipose tissue depots (Zahorska-Markiewicz et al., 2007, Derosa et al., 2013, Sitticharoon et al., 2014, Nourbakhsh et al., 2015). Nevertheless, there are known to be significant links between visfatin and inflammation, endothelial dysfunction and

atherosclerosis (Hognogi and Simiti, 2016). One *in-vitro* study has shown that recombinant visfatin enhances the production of pro-inflammatory cytokines, IL-1β, TNF-α, and IL-6 in human monocytes (Moschen et al., 2007). Some studies have also found an increased concentration of plasma visfatin in patients with type 1 diabetes mellitus (Haider et al., 2006), type 2 diabetes mellitus (Chen et al., 2006a), coronary artery disease (Hognogi and Simiti, 2016), rheumatoid arthritis (Otero et al., 2006) and OA (Laiguillon et al., 2014). Moreover, in dogs with acute pancreatitis or diabetes mellitus, serum visfatin concentrations were to be found significantly higher than in healthy dogs (Paek et al., 2014, Kim et al., 2015). Therefore, visfatin may be more related to obesity-induced inflammation than to fat accumulation and can be considered as a pro-inflammatory adipokine (Friebe et al., 2011).

1.3 The stifle (knee) joint in the dog

The stifle is located in the pelvic limbs of quadruped mammals, including dogs, and is equivalent to the human knee joint (Gupte et al., 2007). The dog is a digitigrade that walks on four digits thus some joint function and movement varies from ungulates and humans (Gupte et al., 2007). The stifle joint is the largest synovial joint and one of the most complex biomechanical systems in the body (Carpenter and Cooper, 2000). The anatomical structure of the stifle joint is composed of different parts: bones, cartilage, ligaments, synovial fluid and infrapatellar fat pad.

1.3.1 Bones and cartilage

The stifle joint contains the articulations of three bones: the distal femur, the proximal tibia and the patella (Robbins, 1990). The ends of these bones are covered with articular cartilage that help the bones move smoothly across each other during flexion and extension (Arnoczky, 1993). Two C-shaped fibrocartilaginous discs that sit between the femoral condyles and the tibial plateau are the medial and the lateral menisci (Arnoczky and Warren, 1983) (Figure 1.1). In man, the menisci primarily perform a load-transmitting function of more than 50% of the compressive load to which the knee joint is subjected during weight bearing (Bessette, 1992). Therefore, they act as shock absorbers and assist with lubrication between the femur and the tibia (Clark et al., 1999). They also improve joint stability by deepening the articular surface of the tibial plateau in dogs (Robbins, 1990, Arnoczky, 1993).
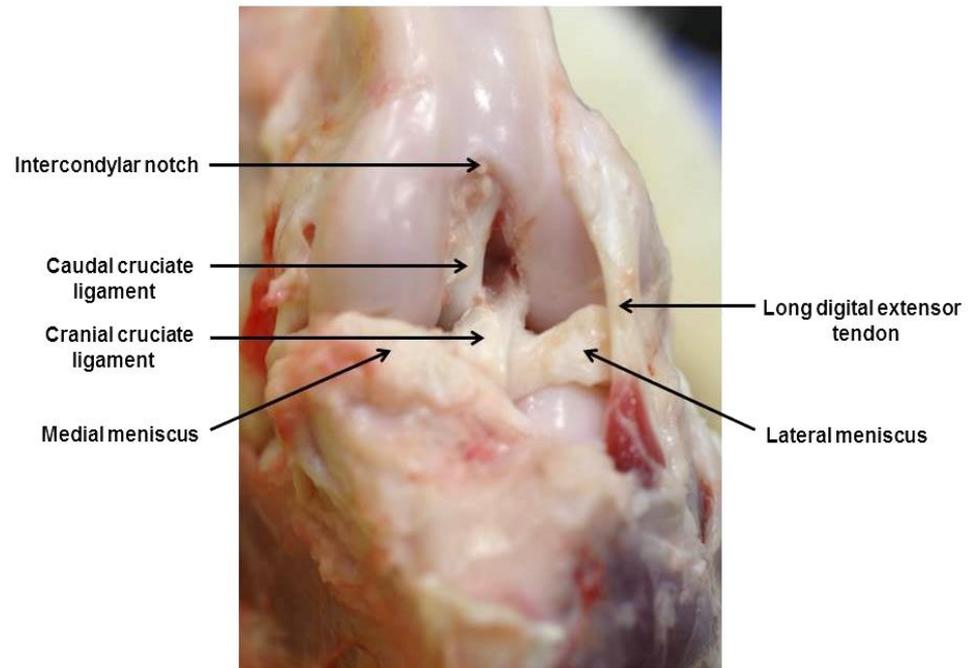
Figure 1.1 Cranial view of a canine stifle joint.

The figure shows cranial view of a canine stifle joint, which is composed of intercondylar notch, cranial and caudal cruciate ligament, medial and lateral meniscus and long digital extensor tendon (Adapted from Allaith, 2016, with permission (Thesis, University of Liverpool)).

1.3.2 Ligaments of the stifle

Ligaments are tough bands of fibrous tissue that attach one bone to another and stabilise joints. They are composed of longitudinal collagen fibres and spindle-shaped cells called fibrocytes or ligamentocytes (Amiel et al., 1984, Vasseur et al., 1985). Collagen, glycosaminoglycans (sGAG) and elastin are the key components in the extracellular matrix of the canine cruciate ligament (Comerford et al., 2005, Smith et al., 2014, Kharaz et al., 2016). Type I collagen is the predominant collagen found in ligaments (> 85%) and is consequently related to mechanical strength of ligaments (Liu et al., 1995). The large aggregating proteoglycans such as decorin and biglycan also play a role in viscoelasticity and act as a lubricant between collagen fibres in ligaments (Amiel et al., 1995).

There are four ligaments that primarily stabilise the canine stifle joint: cranial cruciate and caudal cruciate ligaments and medial and lateral collaterals (Arnoczky and Marshall, 1977). The cruciate ligaments are located inside the stifle joint (Figure 1.1). They cross each other with the cranial cruciate ligament (CCL) (anterior cruciate ligament; ACL, in humans) at the front of the joint and the caudal cruciate ligament (posterior cruciate ligament in humans) at the back (Arnoczky and Marshall, 1977). The CCL arises from a fossa on the caudomedial aspect of the lateral femoral condyle, and some of the fibres come from the caudolateral aspect of the intercondylar area. It then tightens, spirals passing cranially, medially, and distally to insert in a fan-like fashion primarily on the cranial intercondyloid area of the tibia (Heffron and Campbell, 1978, Stouffer et al., 1983). The canine CCL can also be divided into two distinct bundles distinguished from individual attachment sites, the craniomedial band and the larger caudolateral band which spirals laterally between attachments (Arnoczky and Marshall, 1977). The orientation of the CCL prevents the tibia from shifting forward relative to the femur (Arnoczky and Marshall, 1977). The caudal cruciate ligament originates from the intercondylar notch on the lateral side of the medial femoral condyle. The ligament runs caudodistally crossing medial to the CCL and inserting on the medial aspect of the popliteal notch of the tibia. The caudal

cruciate ligament functions to prevent caudal displacement of the tibia in relation to the femur. Moreover, both cruciate ligaments also control the stifle joint from over-extension and limit excessive internal rotation of the tibia during flexion (Arnoczky and Marshall, 1977). In addition, there are two collateral ligaments on the medial and lateral side of the stifle joint. The medial collateral ligament passes distally from the medial femeral epicondyle, and inserts along the medial part of the tibia. The lateral collateral ligament originates on the lateral femoral epicondyle and passes distally to the insertion point on the fibular head (Canapp, 2007). These collateral ligaments are essential for the stability of the joints, chiefly in preventing the lateral displacement of their connecting bones (Arnoczky et al., 1977).

1.3.3 Synovial fluid

The joint cavity is filled with synovial fluid, which is produced from the inner layer or synovial membrane of the joint capsule (Leeson et al., 1988). Normal synovial fluid is clear and light yellow being primarily composed of water and polymerised hyaluronic acid (Leeson et al., 1988). The function of synovial fluid is to act as a lubricant and to supply nutrients to articular cartilage and menisci (Clements, 2006). Mononuclear cells and neutrophils from the blood can penetrate through the synovial membrane helping remove normal degradative products of the articular cartilage and phagocytosing foreign material such as bacteria or fungi (Leeson et al., 1988, Evans and Hermanson, 1993). Many enzymes found in synovial fluid have been used as biomarkers to determine joint disease in companion animals and humans (Hegemann et al., 2003, Catterall et al., 2010). Therefore, evaluating synovial fluid can be important in the examination of joint health (Clements, 2006).

1.3.4 Intra-articular fat

The infrapatellar fat pad (IPFP), also known as the Hoffa fat pad, is located between the between synovial membrane and the joint capsule within the knee joint (Saddik et al., 2004). It mainly covers the anterior part of the knee, just under the

patella (Saddik et al., 2004). IPFP has both protective and disease-enhancing effects in the knee joint (Ioan-Facsinay and Kloppenburg, 2013). For example, it functions as a local shock-absorber and a larger IPFP can reduce lateral tibial cartilage volume loss and knee pain in man (Teichtahl et al., 2015). On the other hand, IPFP primarily consists of adipose tissue, which is similar to SC adipose tissue and can secrete multiple inflammatory cytokines, adipokines and growth factors such as TNF-α, IL-1β, IL-6, leptin and vascular endothelial growth factor (Clockaerts et al., 2012). Moreover, chemical secreted from IPFP conditioned-medium and IPFP adipocyte themselve can induce dedifferentiated and inflammatory phenotypes in articular chondrocytes (Clockaerts et al., 2012, Chen et al., 2016). Therefore, it may play a role in the inflammatory processes in OA.

1.4 Anterior and cranial cruciate ligament disease

1.4.1 Introduction

In human, the anterior cruciate ligament (ACL) is the most commonly injured knee ligament susceptible to tears causing pain, joint instability and eventually degenerative joint disease (Griffin et al., 2000, Murray et al., 2013). An ACL injury can be a result of direct contact with an object to the lower extremity; however, it has been stated that up to 70% of ACL injuries result from noncontact (Boden et al., 2000). Hundreds of thousands of ACL injuries occur in people every year, especially in athletes between 15 and 25 years of age (Griffin et al., 2006). ACL injury is the most common musculoskeletal injury predisposing to OA (Jomha et al., 1999, Brooks et al., 2005, Alentorn-Geli et al., 2009). In the United States, at least 200,000 ACL reconstructions are performed each year with direct costs of approximately $3 billion annually (Brophy et al., 2009). ACL reconstruction surgery has also become a frequent procedure in the UK (Jameson et al., 2012). The aim of ACL reconstruction is to improve stability of a mechanically unstable knee but, unfortunately, it cannot protect against the development of post-traumatic OA and subsequent re-rupture of the ligament (Frosch et al., 2010).

Animal models for the knee joint have been studied in various species including rabbit, dog, pig, sheep, and cow (Murray et al., 2013). Arguably, the dog is most suitable because dog CCL is most like the human ACL in terms of histological appearance (Murray et al., 2004). Canine cranial cruciate ligament disease (CCLD) is a condition whereby progressive degeneration of the CCL leads to ligament rupture similar to non-contact injury in men (Grindstaff et al., 2006). Comparable to humans, CCLD is one of the most common causes of pelvic limb lameness in dogs, which has a huge economic impact for its treatment and management (Wilke et al., 2005, Witsberger et al., 2008) and increased in incidence over the last 40 years from 1.81% to 4.87% (Witsberger et al., 2008). The condition can also lead to consequent meniscal injuries and OA (Bennett and May, 1991, Vasseur and Berry, 1992). Micro-

injury or mechanical loading has traditionally been attributed to traumatic CCL either complete or partial rupture (Griffon, 2010). However, most CCL ruptures in dogs occur spontaneously from progressive and irreversible degenerative alterations of the ligament itself (Hayashi et al., 2003, Lampman et al., 2003). Moreover, nearly 50% of dogs with CCLD will have their contralateral limbs develop CCL deficiency later in their life (Buote et al., 2009).

1.4.2 Risk factors

Risk factors for CCLD include genetics, poor limb conformation, age, reproductive status (being female and neutered), excessive exercise, greater bodyweight and obesity (Witsberger et al., 2008, Inauen et al., 2009, Ragetly et al., 2011). There is a greater prevalence of CCLD in large breeds of dogs, such as Akitas, Labrador Retrievers, Mastiffs, Neopolitan Mastiffs, Newfoundlands, Rottweilers, Staffordshire Bull Terriers and St Bernards (Whitehair et al., 1993, Witsberger et al., 2008, Adams et al., 2011), and a lesser prevalence in other breeds such as Basset Hounds, Dachshunds and old English sheepdogs (Whitehair et al., 1993, Duval et al., 1999). Several genomic studies have identified chromosomal regions and genetic markers which may play a role in non-traumatic canine CCLD particularly in breeds such as the Newfoundland (Wilke et al., 2009, Baird et al., 2014). Poor conformation of the pelvic limb could also predispose to the risk of CCL deficiency (Inauen et al., 2009, Mostafa et al., 2010). In these studies, dogs with a greater inclination of the proximal tibia relative to the distal tibial axis, as well as dogs with a small tibia tuberosity, are predisposed to CCL deficiency because of excessive rotational forces placed on the CCL during weight bearing (Inauen et al., 2009, Mostafa et al., 2010). In addition, many studies have shown that older dogs tend to develop CCLD compared to those of a younger age (Witsberger et al., 2008, Adams et al., 2011). In terms of reproductive status, female dogs and neutered dogs are more likely to have CCLD (Whitehair et al., 1993, Adams et al., 2011). Furthermore, many demographic studies have demonstrated that CCLD is frequently seen in dogs that are overweight

or have obesity up to 4-fold (Whitehair et al., 1993, Brown et al., 1996, Diez and Nguyen, 2006, Adams et al., 2011).

1.4.3 Aetiopathogenesis for CCLD

Mechanical injury may not be the only reason for CCLD, with other mechanisms implicated such as immune-mediated disease, inflammatory cytokines and enzymes (Cameron et al., 1994, Lawrence et al., 1998, Lemburg et al., 2004, Muir et al., 2006, Erne et al., 2009). Pro-inflammatory cytokines are implicated in the pathogenesis of human ACL because IL-1, IL-6, IL-8 and TNF-α, concentrations are increased in the synovial fluid of the knee joint in people with ACL injury (Cameron et al., 1994, Irie et al., 2003). However, it is not known whether these cytokines are secondary consequence of ligament injury or might itself be an inciting cause of ligament degeneration (Comerford et al., 2011). Matrix metalloproteinases (MMPs)-1, -2, -3, -9, -13 all play important roles in matrix degradation; all of these have been associated with the development of OA and are also reported to be the most influential MMPs in CCLD (Comerford et al., 2004, Muir et al., 2005, Sardari et al., 2011, Boland et al., 2014, Haslauer et al., 2014). Increased collagen and glycosaminoglycan (sGAG) synthesis in the extracellular matrix of ruptured CCLs, together with increased sGAG concentration in synovial fluid of dogs with CCLD have been demonstrated (Arican et al., 1994, Comerford et al., 2004). This suggests that there is increased matrix turnover and degradation within the ligament tissue prior to or following rupture.

1.4.4 Clinical assessment of canine CCLD

1.4.4.1 Orthopaedic examination

A thorough orthopaedic examination includes palpation and manipulation of all joints, conformation evaluation and gait assessment (Malikides et al., 2007). CCLD leads to stifle joint instability due to several types of mechanoreceptors found within

the CCL (Georgoulis et al., 2001) .Palpation of the stifle joint should be performed to determine joint effusion)Jerram and Walker, 2003 .(Periarticular thickening around the medial surface of the joint, also known as the buttress sign, is often seen and is believed to be associated with chronic meniscal injury (Piermattei et al., 2006). Palpation of both pelvic limbs can also be used to identify muscle atrophy in comparison with the contralateral limb (Jerram and Walker, 2003). The cranial drawer sign, using direct (cranial drawer test) and indirect (tibial compression test) palpation techniques can provide a definitive diagnosis for CCLD (Piermattei et al., 2006), where the tibia can be displaced cranially (Moore and Read, 1996a).

With partial CCL rupture, a dog presenting with hind limb lameness but no obvious drawer sign is a common situation. Synovial fluid analysis, computed tomographic arthrography and magnetic resonance imaging should be considered to evaluate joint inflammation, and to help diagnose the damage of cruciate ligament fiber (Faldyna et al., 2004, Han et al., 2008, Erne et al., 2009, Sample et al., 2017).

1.4.4.2 Subjective lameness evaluations

Dogs with CCLD typically show pelvic limb lameness due to pain and instability in the associated stifle joint (Jerram and Walker, 2003). Dogs with CCLD may also have meniscal tears at the time of injury but this can also occur later, leading to pain and increased lameness (Wustefeld-Janssens et al., 2016). The severity of lameness can vary from non-weight-bearing to intermittent, depending on the time since injury and degree of damage to the CCL and menisci (Piermattei et al., 2006). There are several subjective and objective methods to assess severity of lameness in dogs (DeCamp, 1997, Lane et al., 2015). However, a numerical rating scale system and a continuous scale are commonly used in general practice because it is convenient and repeatable (Hudson et al., 2004, Mathews et al., 2014). Veterinarians can easily use this system to grade degree of lameness into categories from 0 (clinically sound) to 5

points or 10 points (continuous non-weight bearing) when viewing the dog at a walk and trot (Vasseur and Slatter, 1993, Malikides et al., 2007, Mathews et al., 2014).

1.4.4.3 Objective lameness evaluation

Objective observations of gait analysis, such as kinetic (force plate analysis) and kinematic evaluation (computer-assisted three-dimensional analysis, electromyography) are more reliable than visual gait assessment since they eliminate human observer bias (Quinn et al., 2007). These objective measurements of limb function are the gold standard for determining lameness (DeCamp, 1997, Gillette, 2004). Many veterinary orthopaedic studies, including those on canine CCLD, have used force plate analysis for assessing lameness (Ballagas et al., 2004, Brown et al., 2013). A normal gait cycle includes a stance phase, which is the period when the foot is in contact with the ground, and a swing phase where the foot is in the air (DeCamp, 1997). During each stance phase, force plates connected to computers quantify the three orthogonal ground reaction forces (GRFs) as the dog walks over the plate, which include vertical (F_z), craniocaudal (F_y) and mediolateral (F_x) forces (Figure 1.2A). These forces are then generated into pressure graphs on a computer screen for each strike and can be used for analysis with specific software (Figure 1.2B) (DeCamp, 1997, Gillette and Angle, 2008). F_z most directly measure weight-bearing, have the greatest magnitude compared with other orthogonal forces and are good parameters for asymmetry assessment of left and right limbs (DeCamp, 1997, Fanchon and Grandjean, 2007). Therefore, F_z variable such as peak vertical force (PVF), vertical impulse (VI), rising slope (RS) and falling slope (FS) as well as symmetry or asymmetry indices (AI) have been used for detection of pelvic limb lameness in dogs (Fanchon and Grandjean, 2007). At walk, the first peak of F_z corresponds to the PVF allied with the initial paw strike in early stance and the second peak, if present, represents maximal vertical forces generated in late stance associated with toe-off or propulsion (DeCamp, 1997) (Figure 1.2B). At trot, F_z in the dog are graphed as single, sharper peaks for both forelimbs and rear limbs, because the stance phase

happens more quickly (Figure 1.2c). Obese dogs normally have greater PVF and horizontal GRFs than lean dogs (Brady et al., 2013). PVF and VI are the most commonly used parameters measured in the assessment of pelvic limb lameness in veterinary orthopaedic studies. These parameters are reduced in value in dogs with CCLD or when lameness is present (Budsberg et al., 1988, Jevens et al., 1996, Ballagas et al., 2004, Voss et al., 2008).

RS is defined as the slope of the straight line that connects the beginning of the stance phase to the first peak and represents the rate at which a dog loaded the limb on the plate. Conversely, FS is defined as the slope of the straight line that connects the peak to the end of the stance phase which represents the rate at which the dog unloads the limb off the plate (Evans et al., 2005). Pelvic limb lameness usually causes a reduction in mean rising slope and an increase in mean FS (Budsberg et al., 1988, Budsberg et al., 1996).

In healthy dogs, there is no significant asymmetry between the 2 body sides of locomotion detected during walking and trotting (Budsberg et al., 1993, Fischer et al., 2013). Conversely, when lameness is present, the load decrease in the injured limb and the compensatory load increase in the contralateral limb results in an alteration in limb symmetry (Böddeker et al., 2012, Fischer et al., 2013). Therefore, the parameters from force plate measurement can help in the investigation of abnormalities in a step cycle of gait, and are also useful in assessing medical or surgical treatment outcomes (Voss et al., 2008, de Medeiros et al., 2011, Vassalo et al., 2015).
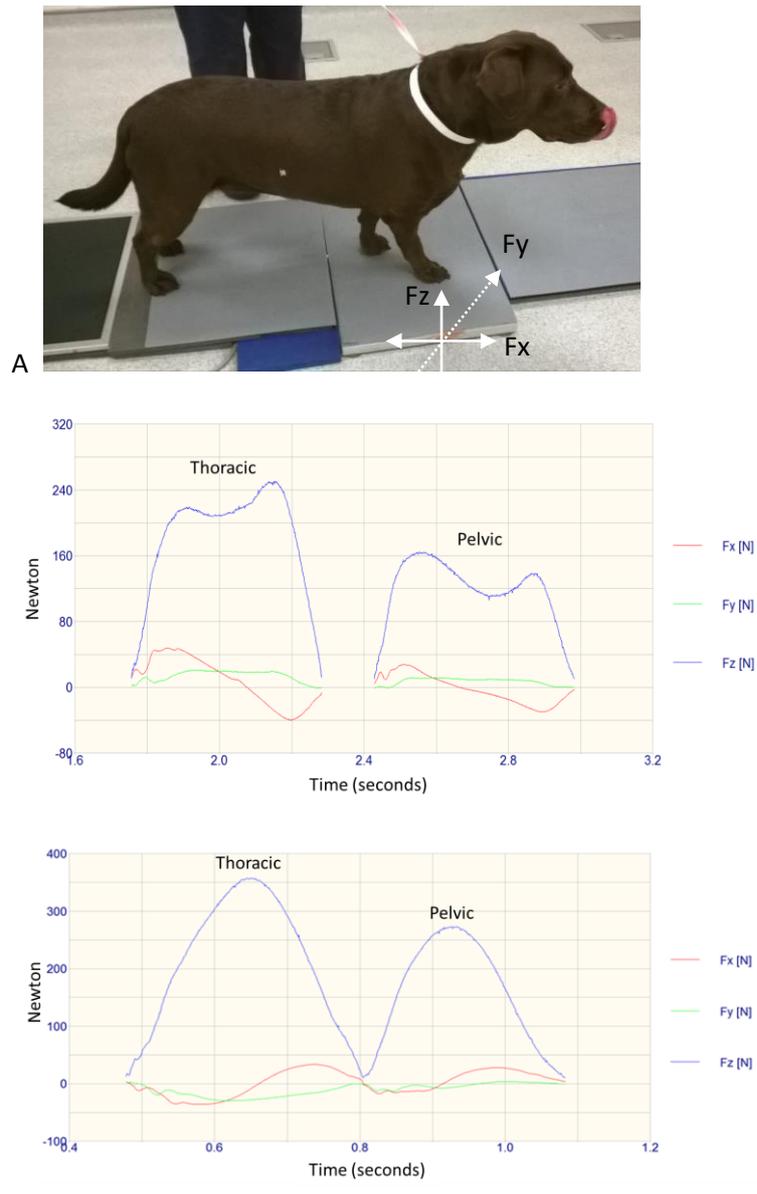
Figure 1.2 Kinetic force plate analysis of limb function.

Figures show three orthogonal ground reaction forces; vertical (Fz), craniocaudal (Fy) and mediolateral (Fx) are detected as the dog steps on a force plate (A). The force reactions of two limbs; the left graph and the right graph display forces from a pelvic limb and a thoracic limb at walk (B) and at trot (C). The blue, the green and the red line represent data points associated with Fz, Fy and Fx force respectively.

1.4.4.4 Radiographic assessment of the stifle joint

Imaging of the canine stifle joint using radiography is helpful for assessing the affected joint, to evaluate severity of OA secondary to CCLD and to exclude other musculoskeletal abnormalities such as fractures and/or neoplasia (Jerram and Walker, 2003, Böddeker et al., 2012). Common radiographic findings in cases of CCLD include muscle atrophy, joint effusion, periarticular swelling and periarticular osteophyte formation (Piermattei et al., 2006). In rare cases, ligament avulsions can be seen (Piermattei et al., 2006). Previous studies have shown that CCLD results in stifle joint instability and initiated the development of stifle OA (Vasseur and Berry, 1992). OA pathogenesis is characterised by an increased thickness of the subchondral bone, destruction of articular cartilage, osteophyte formation, synovial hyperplasia and fibrosis of the joint capsule (Gilbertson, 1975, Dedrick et al., 1993). Therefore, radiographic grading systems have been developed for the canine stifle joint and have been regularly performed in CCLD cases to evaluate degeneration of the joint (Innes et al., 2004, Lazar et al., 2005). Other imaging techniques used in the diagnosis of meniscal disease include ultrasonography, magnetic resonance imaging, plain and contrast computed tomography and arthroscopy (Gnudi and Bertoni, 2001, Barrett et al., 2009, Samii et al., 2009, Plesman et al., 2013). However, these modalities require specialist equipment and skilled operators, which can result in low specificity and sensitivity (Bergman et al., 2007).

1.4.5 Treatment of CCLD

There are several factors that veterinarians must consider when selecting the most appropriate treatment option for canine CCLD including severity of lameness, age, bodyweight and the equipment available along with expertise in its use (Comerford et al., 2013, Duerr et al., 2014). The aims of treatment are to improve stability and to try and restore the normal functions of the stifle joint (de Rooster et al., 2006). Treatment options for CCLD included conservative and surgical management (Jerram and Walker, 2003). Conservative management is reportedly a reasonable option for managing CCLD in dogs under 15 kg bodyweight (Comerford et

al., 2013), and options include controlled weight loss, the administration of analgesic medications, physiotherapy and rest. There are many surgical techniques for managing CCLD including extra- and intra-capsular techniques and tibial osteotomies such as tibial plateau levelling osteotomy (TPLO), tibial tuberosity advancement (TTA), and triple tibial osteotomy (TTO) (Innes, 2012, Bergh et al., 2014, Knebel and Meyer-Lindenberg, 2014). However, despite a range of surgical methods, the most effective treatment for CCLD remains controversial because none of them have been found to be effective in stabilising the joint and minimising the degeneration of the joint (Lazar et al., 2005, Krotscheck et al., 2016). Extracapsular stabilisation methods have been recommended for use in small breed dogs (Duerr et al., 2014); however, there is little good quality evidence to suggest that these techniques do not work in larger dogs (Chauvet et al., 1996). Such techniques involve the use of heavy-gauge suture such as leader line nylon or fibre wire to decrease joint instability by attaching with soft or bony tissues (Cook et al., 2010). Intracapsular procedures substitute the function of the ruptured CCL with an autograft, allograft, xenograft or synthetic materials; however, these techniques have not been shown to be effective (Person, 1987, Moore and Read, 1996b). TPLO is one of the most common surgical methods for CCLD management in large and small breed dogs (Duerr et al., 2014). Unlike in man, the canine tibial plateau has an increase slope on cranial part; so the purpose of an osteotomy is to level it (Slocum and Slocum, 1993). TPLO aims to make the tibial plateau perpendicular to the long axis of the tibia. Therefore, neutralise the mechanical forces acting on the stifle, rendering the cranial cruciate ligament unnecessary (Kowaleski and McCarthy, 2004).

The TTA is a less invasive surgery and gives similar outcomes to the TPLO (Duerr et al., 2014). The procedure involves making a cut in the front part of the tibial tuberosity and advancing this portion of bone forward. The tibial tuberosity is secured with specially designed implants so that the angle of the patellar ligament approaches 90 degrees to the tibial slope and the opposing forces become

eliminated. Thus, the tibia bone remains in place when weight is placed on the limb (Lafaver et al., 2007).

1.4.6 Post-operative management and assessment of CCLD

Post-operative management and follow-up examination for CCLD are important in overall treatment outcome. Several studies have indicated that controlled weight loss significantly improves clinical signs and limb function of dogs with CCLD and OA (Impellizeri et al., 2000, Mlacnik et al., 2006, Wucherer et al., 2013). Therefore, educating clients of overweight dogs about weight loss should be considered both pre- and post-management for CCLD (Innes, 2012). In terms of exercise programmes as post-surgical management of CCLD, some veterinarians are hesitant to promote early use of the limb and instead advise exercise restriction (Comerford et al., 2013). However, excessive immobility of the joint could cause adverse biochemical and metabolic changes in articular cartilage (Keller et al., 1994). Recent studies have shown a well-designed and frequent rehabilitation programme performed in the initial post-operative period was safe and encouraged rapid return to normal limb function for dogs (Marsolais et al., 2002, Romano and Cook, 2015). In general, a complete clinical orthopaedic examination, mediolateral and caudocranial radiographs of the operated stifle joint are performed during the follow-up examinations at 6 to 8 weeks depending on which surgical option has been used (Jerram and Walker, 2003). Kinetic and kinematic gait analyses are also recommended for assessing return of joint function at this first follow-up if accessible (DeCamp, 1997, Voss et al., 2008). A minimum long-term follow-up time of 6 months is suggested; whilst longer-term follow-up could also be important (Bergh et al., 2014). This is because alteration of biomechanics within the stifle joint after surgical intervention may have long-term effects on eventual clinical outcomes (Bergh et al., 2014).

1.5 Link between adipokines and joint diseases

Obesity is suggested to be a risk factor for musculoskeletal injuries in humans (Heir and Eide, 1996), and especially lower extremity joint disorders (Andriacchi et al., 2004), with the excess bodyweight from obesity causing increased mechanical loading. Indeed, the risk for knee injury increases with increasing BMI in humans (Sulsky et al., 2000, Webb et al., 2004, Mattila et al., 2007); obese men (BMI > 30) have a threefold higher risk of medial and lateral collateral ligaments tears (Sillanpaa et al., 2008), a twofold increase in the risk for patellar dislocation (Kuikka et al., 2013), and an increase in the incidence of the medial meniscal tear compared with overweight men with lower BMI (Ozkoc et al., 2008).

However, several human studies have also identified obesity as an independent risk factor in non-weight-bearing joints such as the hand and upper extremity soft tissue (Carman et al., 1994, Cicuttini et al., 1996, Dahaghin et al., 2007, Rechardt et al., 2013). Other studies have shown that some joint diseases such as rheumatoid arthritis (RA) and OA are associated with both adipose mass and central adiposity (Otero et al., 2006, Wang et al., 2009). Moreover, it has been shown that a reduction in body fat is more advantageous than body mass loss in relieving of OA progression (Toda et al., 1998). Therefore, it is suggested that other mechanisms rather than increased mechanical loading might be responsible for the adverse effect of adipose tissue in musculoskeletal disease. For example, as discussed above, adipose tissue is a major source of adipokines and inflammatory cytokines, and these might instead be provoking cartilage and articular tissue degeneration (Doom et al., 2008, McNulty et al., 2011). In this respect, recent studies have demonstrated that the IPFP is a main source of adipokines and cytokines produced in human knee (Ushiyama et al., 2003, Presle et al., 2006, Klein-Wieringa et al., 2011). Further, gene expressions of leptin and visfatin are increased in the IPFP in mice fed on a high fat diet and are correlated with expression of inflammatory cytokines such as TNF-α in the IPFP (Iwata et al., 2013). Serum leptin concentration in mice fed with a high-fat diet increase with OA severity, whereas mice with leptin deficiency can be morbidly

obesity but have no signs of OA (Griffin et al., 2009, Griffin et al., 2010). These studies suggest that adipokines might have local effects in the development of joint inflammation and degeneration.

In human, adiponectin, leptin and visfatin are also implicated in different joint diseases such as OA and RA (Lago et al., 2007a). Recent investigations into the role of adipokines in the development of OA have demonstrated that plasma adiponectin concentration and adiponectin released from knee cartilage of OA patients are positively correlated with their knee radiographic scores (Koskinen et al., 2011). Moreover, adiponectin also induces production of nitric oxide, IL-6, MMP-1 and MMP-3 in primary chondrocytes and in OA cartilage *in vitro* (Koskinen et al., 2011). Gene expression and protein concentration of leptin in osteophytes, cartilage and synovial fluid from patients with knee OA are found to be higher than in a healthy control group (Dumond et al., 2003, Ku et al., 2009). One study demonstrated an association of leptin concentration in synovial fluid and the radiographic severity of knee OA (Ku et al., 2009), whilst a second study suggested that the adiponectin-to-leptin ratio in synovial fluid could predict knee pain in subjects with knee OA (Gandhi et al., 2010). Leptin is reported to have a catabolic effect on chondrocyte metabolism by inhibiting chondrocyte development, inducing production of pro-inflammatory cytokine (IL-1) and up-regulating expression of matrix metalloproteases such as MMP- 9 and -13 (Gualillo, 2007, Simopoulou et al., 2007, Hui et al., 2012).

In addition, a study on RA has found evidence of increased adiponectin, leptin, and visfatin concentration in plasma of the patients (Otero et al., 2006). The concentrations of visfatin in serum and synovial fluid have also been found to be correlated with radiographic joint damage, degree of inflammation and matrix-degrading activities in the joints of RA patients (Brentano et al., 2007, Rho et al., 2009). A recent study has also shown that visfatin may promote the catabolic degradation of both cartilage and meniscus *in vitro* (McNulty et al., 2011). From this evidence, it is suggested that adiposity in the body may have biological effects on joint degeneration (Wang et al., 2015). However, local adipokines produced from

intra-articular fat are considered as humoral factors that can contribute to the development and progression of various joint diseases such as in OA and RA (Toussirot et al., 2007).

An increased fat mass and percentage of fat of lower extremity has been observed in patients with unilateral ACL disease as compared to the intact lower extremity (Takata et al., 2007). In dogs, obesity has been described as a risk factor for CCLD (Adams et al., 2011). Joint inflammation induced by biochemical factors such as cytokines has been implied in CCLD (Erne et al., 2009). Nonetheless, the precise mechanisms underlying the association between humoral effect of obesity and CCLD have not yet been clarified. In this regard, studying the role of adipokines in canine CCLD may be essential in developing future preventative strategies and management of this condition in the dog and may act as a relevant model for human disease. A better understanding of the disease mechanisms would also enable us to possibly target the degenerative ligament processes, which may lead to a reduction in the incidence of non-contact cruciate rupture and OA in both dogs and humans.

1.6 Hypothesis and Aims

1.6.1 General Hypotheses

The hypotheses of the study are that both systemic and local (intra-articular stifle joint tissues) cross-talk occur between adipokines and articular tissue in obese dogs and that mechanisms can play a role in the pathophysiology of CCLD.

1.6.2 Aims

To answer these hypotheses, this study aimed to:

- Investigate the relationship between mechanical (bodyweight) and humoral (body fat composition) factors of obesity and clinical presentation of dogs with CCLD.

- Examine differences in gene expression of pro-inflammatory cytokines, tissue degradation biomarkers, and adipokines in dogs with and without CCLD.

- Determine the association of both local and systemic adipokines with pro-inflammatory cytokines and tissue degradation biomarkers in CCL tissues and synovial fluid of dogs with CCLD.

- Investigate the relationship between chemical mediators in synovial fluid and CCL tissues of the stifle joint and clinical presentation in dogs with CCLD.

- Examine cross-talk between adipose tissues (both systemic and local) and CCL tissues on CCL explants in terms of glycosaminoglycan production using co-culture systems.

- Examine the effects of adipokines (adiponectin, leptin, and visfatin) on CCL cells in relation to cartilage degradation biomarkers gene expression, cells viability and cells migration activities.

Chapter 2 Materials and Methods

2.1 Animals

2.1.1 Control group

Dogs that had been euthanased for reasons other than musculoskeletal disease were used as a control group. Ethical permission for using such material was provided following local institutional ethical review (RETH0000553 and VREC213). Signalment information including dog breed, sex, age and bodyweight were collected. Samples including synovial fluid, cranial cruciate ligaments (CCLs), infrapatellar fat pad (IPFP) were harvested from the right stifle joint and subcutaneous (SC) fat from the inguinal area of cadaveric dogs within 24 hours of death. Tissues were either snap-frozen in liquid nitrogen, and stored at -80°C until further analysis or processed immediately for tissue culture and cell isolation.

2.1.2 Clinical group

The clinical group for this study comprised dogs diagnosed with cranial cruciate ligament disease (CCLD) referred to the Small Animal Teaching Hospital, University of Liverpool. CCLs were obtained at surgical intervention for the management of CCLD in affected dogs.

Dogs were excluded if they had bilateral CCLD, lumbosacral disease or pain in any other pelvic limb joints. Fully informed written consent was obtained from all owners who allowed their dogs to be recruited in this study. Ethical permission for using these samples was granted following local institutional ethical review (VREC192). Signalment data including dog breed, sex, age and bodyweight were obtained from clinical records. Synovial fluid and tissues including ruptured CCLs, IPFP, SC were also collected from the clinical dog group at the time of arthroscopy/arthrotomy. Tissues were either snap-frozen in liquid nitrogen, and

stored at -80°C until further analysis or processed immediately for tissue culture and cell isolation.

2.2 Body condition score evaluations in control and clinical groups

Body condition score (BCS) was recorded for all animals in both groups. The BCS for each dog was assigned by two investigators, Wipawee Saengsoi (WS) and Alex German (AG), using a modification of the 9-point system based on size of dogs and images in specific category (Laflamme, 1997b). This system describes the body condition of dogs with values between 1 and 9; where BCS scores of 1-3 points are too thin, 4-5 being ideal, 6-7 being overweight and 8-9 being obese (see Appendix 1 and Chapter 1 Section 1.1.4).

2.3 Diagnostic investigations and body fat composition measurements in the clinical group

2.3.1 Lameness score

Subjective lameness scores were graded to all dogs with CCLD by an ECVS resident or RCVS or ECVS Diploma holding orthopaedic surgeon from the Small Animal Teaching Hospital orthopaedic service using a Likert scale of 0 – 10. This scale ranges from 0 (sound at all times) to continuous, non-weight bearing lameness being 10 (Vasseur and Slatter, 1993).

2.3.2 Force plate measurement

Objective measurements of lameness were determined using kinetic gait analysis as previously described (Chapter 1 Section 1.4.4.3). Gait analysis was performed immediately pre-operatively. Dogs were walked at an average speed of

1.0-1.3 m/s over a force platform (Kistler, Winterthur, Switzerland) situated in the middle of a 10-metre runway. Data including peak vertical force (PVF), vertical impulse (VI), falling slope (FS), together with asymmetry indices of these values were collected electronically using Qualisys QTM (Gothenburg, Sweden) and Bioware (Kistler) software. Five replicates of these parameters were analysed in a computer spreadsheet (Excel, version 14.0.6106.5005, Microsoft Office Professional Plus 2010) (Evans et al., 2005, Fanchon and Grandjean, 2007). Synchronised digital video files were also collected and stored to validate each dog's gait cycle.

2.3.3 Radiographic examination and score

Orthogonal radiographic views (mediolateral and caudocranial) were obtained under sedation in all dogs in the clinical group to confirm the diagnosis of CCLD as well as for surgical planning. Radiographic scores were determined by examination of the mediolateral and caudocranial views (see Appendix 2) of the index stifle joint according to a 5-point Likert system, with 0 representing no osteophytosis and 4 representing the most severe osteophytosis (Innes et al., 2004). Radiographic views were independently scored twice by two blinded investigators, WS and Eithne Comerford (EC), at least two weeks apart.

2.3.4 Dual-Energy X-Ray Absorptiometry (DEXA)

To determine body fat composition, each dog was scanned using a fan-beam DEXA scanner (Lunar Prodigy Advance, GE Lunar, Madison, USA) while the dogs were sedated for radiographic examination (Section 2.3.3) or prior to surgical management for CCLD. The scanner performed a single sweep, with the dog positioned in dorsal recumbency on the scanner table (Figure 2.1A). A whole-body scan was generally completed in 4-6 minutes depending on the dog's breed. The manufacturer's software (enCORE 2004, 8.70.005; GE Lunar) was then used to analyse the resulting images (Figure 2.1B).

A   B

Figure 2.1 Dual-energy X-ray absorptiometry (DEXA) scanning.

Figures show a dorsal recumbency position of the dog during DEXA scanning (A) and a typical example of an image obtained from the scan (B). In figure B, lines determine regions of interest used to calculate body fat mass, grey areas show air and white areas show muscle, fat and bone.

2.4 Tissue culture

2.4.1 Tissue explant culture

CCL (approximately 50 mg), IPFP (approximately 150 mg) and SC (approximately 150 mg) explants were dissected from the whole tissue samples collected from control and clinical dogs (as described in Section 2.1.1-2.1.2). Explants were cultured in five different groups; CCL, IPFP, SC, CCL+IPFP, CCL+SC, in complete media containing Dulbecco's Modified Eagle's Medium (Gibco®, Life Technologies, MD, USA), 10% foetal bovine serum (FBS, Sigma-Aldrich), 100 U/ml penicillin-streptomycin (Gibco®) and 2 µg/ml amphotericin B (Gibco®). Media from the culture were collected and replaced every 3-4 days.

2.4.2 Cell isolation

Fresh CCL samples were collected and transported to the laboratory within about 30 minutes (as described in Section 2.1.1-2.1.2). The sample was placed in a Petri dish and finely chopped using a number 20 disposable scalpel before being transferred into a 15-ml falcon tube containing freshly prepared digestion medium which contained collagenase type II (Worthington Biochemical Corporation, Lakewood, USA) in complete media (as detailed in Section 2.4.1) at a concentration 1 mg/ml. This digestion medium was sterilised by filtrating it through a 0.2 µm disposable filter. The tube was shaken horizontally in a 37°C incubator at 170 cycles/minute for 16 hours to digest the tissue (Hwang et al., 2010). After 16 hours, undigested material was removed by passing it through a cell strainer followed by centrifugation (200 x g for 10 minutes) to obtain a cell pellet allowing the supernatant to be removed and discarded using a pipette. The cell pellet was re-suspended in 10 ml of sterile of complete media (as described in Section 2.4.1) followed by centrifugation twice, as detailed above, to wash the cells. Cell count was determined using a haemocytometer (Agar Scientific, Essex, UK) and cells were then seeded into a T75 flask at a density of 2.1 x 10^6 cells per flask. Complete media was added to the

flask making up to a final volume of 15 ml. The cells were kept in an incubator (Sanyo Electric Biomedical, Osaka, Japan) at 37°C in a >98% humidified, 95% air and 5% CO_2 atmosphere.

2.4.3 Maintenance of CCL cells

Primary canine CCL cells were maintained with a complete media (as described in Section 2.4.1) change every 2-3 days until cells reached 80% confluence. For propagation, medium was removed, and the cell layer was washed with phosphate buffered saline (PBS). PBS was removed and 5 ml of 0.05% trypsin in 0.53 mM EDTA (Gibco®) was then added into T75 flask to detach the cells. The cells were incubated at 37°C for five minutes, or until all cells had detached from the flask. An equal volume of serum-containing cell culture medium was next added to stop the cell detachment procedure. After counting the suspended cells with a haemocytometer, cells then were distributed into new flasks for continued expansion or into 24-well plates (10,000 cells/cm^2) for use in future experiments. All cells used in experiments were at the second or the third passage.

In order to freeze cells for future use, cell suspensions were transferred into a 15-ml falcon tube and centrifuged at 200 x g for 10 minutes. Supernatant was removed from pellet; the cell pellet was then re-suspended in cell culture medium and the centrifugation repeated. The cell pellet was then re-suspended in complete medium containing 10% v/v dimethyl sulfoxide (DMSO, Sigma-Aldrich). The cell suspensions (1 ml) were distributed into 1.5-ml cryogenic vials. The vials were put in a cell freezing container (CoolCell®, Biocision, Larkspur, USA) at -80°C overnight before transferring to the -80°C freezer until required.

2.4.4 Cell viability assay

CCL cell viability was analysed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's protocol. This assay determines the number of viable cells in culture based on quantitation of the ATP present (Lovborg

et al., 2002). Briefly, thawed CCL cell samples were cultured in a 96-well flat-bottom white tissue culture plate (VWR, Radnor, USA). at 3,000 cells per well (8,824 cells/cm^2). Cells were treated in triplicate with different concentrations (10 and 50 ng/ml) of recombinant human adiponectin, recombinant human leptin and recombinant human visfatin (Peprotech, Rocky Hill, USA). Cells treated with a toxic dose 200 µg/ml of lipopolysaccharide (Sigma-Aldrich) were used as positive controls (Sharifi et al., 2010) and untreated cells as negative controls. Background luminescence was determined using wells containing medium without cells. After incubation of the cell cultures for 48 hours at 37°C in a >98% humidified, 95% air and 5% CO_2 atmosphere, the culture media in each well was replaced with 100 µl of fresh complete media and topped up with 100 µl with CellTiter-Glo® reagent using a multichannel pipette. The contents were mixed on a plate shaker for two minutes and incubated at room temperature for ten minutes. Luminescence was then recorded using a GloMax® Multi+ Detection System (Promega) (Riss et al., 2004, Niles et al., 2009).

2.4.5 Cell migration assay

For this assay, a protocol developed by Dr. Christopher Mendias's laboratory (Department of Orthopaedic Surgery, University of Michigan, Ann Arbor, USA) was followed based on a collagen gel technique (Hotary et al., 2000, Hudgens et al., 2016). Briefly, type I collagen, derived from rat tail tendons (kindly provided by Dr.Mendias), was dissolved in 0.2% acetic acid at a final concentration of 2.7 mg/ml at 4°C overnight. To induce gelling, collagen was mixed with 10X MEM (Minimum Essential Medium, Gibco®) and 0.34 N NaOH in an 8:1:1 ratio on ice and 350 µl of this mixture was added to the upper well of 12-mm transwell plates (3-µm pore size; Corning®, Sigma-Aldrich). The plates were placed in the incubator at 37°C for one hour. After the gels were set, 1x10^4 CCL cells in 300 µl of complete medium (prepared with 10% heat-inactivated FBS) were added to the upper wells. A further 1.5 ml of complete medium was also added to the lower compartment of the transwell chambers. After an additional 24-hour incubation period, to allow cells to acclimatise, 20 ng/ml of

recombinant human platelet-derived growth factor (PDGF-BB, R&D Systems) was added to initiate cell migration as positive controls and/or 10 ng/ml of adipokines (adiponectin, leptin and visfatin) were added to the cultures. These factors were introduced into the lower compartment of the transwell chambers (Figure 2.2). The medium and chemical reagents were replaced every 2-3 days for 7 days. The collagen gels were then fixed with 4% paraformaldehyde and paraffin-embedded. Sections of the collagen gel were cut at 7 μm, mounted on microscope slides and stained with haematoxylin and eosin (Feldman and Wolfe, 2014). The percentages of migrating cells and maximum migrated distance were quantified under a microscope as the mean ± S.E from five different fields of each gel.
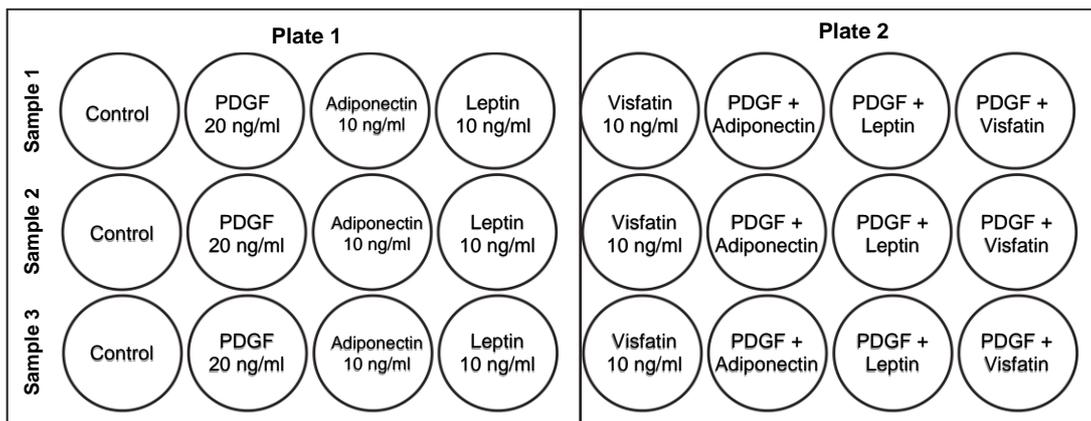
Figure 2.2 Cell migration experiment template.

Cultures of CCL cells isolated from control and clinical dogs (sample 1 - 3) were exposed to 10 ng/ml of adipokines; adiponectin, leptin and visfatin. 20 ng/ml of recombinant human PDGF-BB (PDGF) was used as a positive control.

2.4.6 Glycosaminoglycan measurement

Spectrophotometric assays, based on the colour reaction with 1,9-dimethylmethylene blue (DMMB), were used to measure the glycosaminoglycan (sGAG) content in control and clinical tissues (Farndale et al., 1982, Homer et al., 1993). After 14 days of culture, tissue samples were weighed and digested, with 10 units/ml of papain (Sigma-Aldrich) in 100 mM sodium acetate, 2.4 mM EDTA, 5mM L-cysteine, pH 5.8 in a sufficient volume just to cover the samples in 1.5 ml centrifuge tubes, overnight at 60 °C. The samples were vortex mixed several times during digestion to ensure complete digestion. After tissue digestion method, CCL in papain suspension was diluted 1:10 in deionised water before measuring to ensure sGAG concentrations were within the linear range of the assay. DMMB dye was prepared from 1 L deionised water containing 16 mg DMMB (Sigma-Aldrich), 2 g sodium formate and 2 ml formic acid (Merck, Darmstadt, Germany) at pH 3.5. Then 250 µl of DMMB dye was added to 40 µl of papain digestions or media samples in 96-well plates. Absorbance of the resulting solution was immediately measured using a plate reader (Multiskan FC, Thermo Scientific) at 570 nm. sGAG concentration in each well was calculated by comparison to chondroitin sulphate standards (prepared from shark chondroitin sulphate C, Sigma-Aldrich) in the concentration range of 0 – 70 µg/ml (Figure 2.3), and sGAG concentration was expressed as µg per mg dry weight tissue.
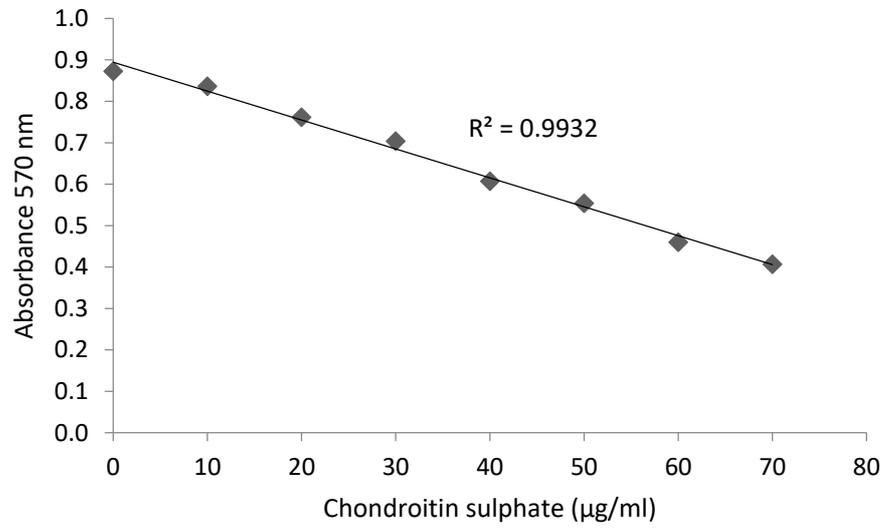
Figure 2.3 Glycosaminoglycan standard curve.

The glycosaminoglycan standard curve generated from the absorbance of chondroitin sulphate in the concentration range of 0 – 70 µg/ml after their reaction with 1,9-dimethylmethylene blue.

2.5 Measurement of the gene expression and protein concentration of cytokines and adipokines in control and clinical groups

2.5.1 Measurement of gene expression

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to determine gene expression for interleukin-6 (IL-6), macrophage chemotactic protein-1 (MCP-1), tumour necrosis factor-α (TNF-α), aggrecan (AGC), type I collagen alpha two chain (COL1A2), matrix metalloproteinase-13 (MMP-13), adiponectin, leptin, and visfatin in tissue samples as described in Section 2.1.

Table 2.1 List of primer sequences used for target genes as designed using Eurogentec, Seraing, Belgium.

Gene	Final concentration (nM)	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
β-actin	300	GCATCCTGACCCTCAAGTACC	AGCTCGTTGTAGAAGGTGTGG
RPS-13	300	GCAGATCTACAAACTGGCCAAGA	TGCAACACCATGGGAGTCTCT
IL-6	600	AAAGAGCAAGGTAAAGAATCAGGATG	GCAGGATGAGGTGAATTGTTGTG
MCP-1	600	TCCTCTGCCTGCTGCTCATAG	GGCCAGCCTCTGAATTGAGATC
TNF-α	300	CCGTGAGAAGATGACCCAGATC	GACAGCACAGCCTGGATGG
AGC	300	GGGACCTGTGTGAGATCGAC	GTAACAGTGGCCCTGGAACT
COL1A2	300	CTATCAATGGTGGTACCCAGTTT	TGTTTTGAGAGGCATGGTTG
MMP-13	300	CCGCGACCTTATCTTCATCT	AACCTTCCAGAATGTCATAACCA
Adiponectin	600	CCGTGATGGCAGAGATGGC	AGCCTCGGGGACCTTCAAC
Leptin	600	AGGATCAATGACATTTCACACACG	GGACAAACTCAGGACTGGTTGG
Visfatin	300	AAGGCCTGTACTGCAATTTTATATGTC	GGAAGCAGAAATTCTCTCTAAAAACATC

RPS-13: ribosomal protein S13; IL-6: interleukin-6; MCP-1: macrophage chemotactic protein-1; TNF-α: tumour necrosis factor-α; AGC: aggrecan, COL1A2: type I collagen; MMP-13: matrix metalloproteinase-13.

2.5.1.1 RNA extraction from stifle and subcutaneous tissues

Approximately 200 mg of the tissue samples (CCL, IPF and SC) collected from control and clinical dogs (as described in Section 2.1.1-2.1.2) were finely chopped and frozen in liquid nitrogen before being powdered using a mikro-dismembrator (Braun Biotech International, Melsungen, Germany). The dismembrator equipment was cooled in liquid nitrogen before use to ensure the tissue remained frozen during the homogenization process. Each sample was shaken at 72 x g for one minute or until the whole tissue become homogenised. One ml of Tri-reagent solution (Ambion, Applied Biosystems, Warrington, UK) was used to digest the homogenised samples at room temperature (20 °C) for 30 minutes before being stored at -80 °C until required.

Using phenol-chloroform extraction technique, once the samples were required they were allowed to thaw, vortex mixed and centrifuged at 20,817 x g for 10 minutes at 4 °C to separate insoluble material from the Tri-reagent extract. To every 1 ml of Tri-reagent, 200 µl of chloroform (Sigma-Aldrich, MO, USA) was added and the sample shaken and left at room temperature for five minutes. The sample was centrifuged again (20,817 x g, 15 minutes, 4 °C) with the clear, upper aqueous phase being carefully removed and transferred to a fresh tube. To this aqueous phase material, an equal volume of 70% ethanol in 0.1% diethylpyrocarbonate (DEPC)-treated dH20 (Sigma-Aldrich) was added (Rio et al., 2010). Total RNA was then purified using an RNeasy mini kit® (Qiagen, Crawley, UK) according to the manufacturer's instructions. Briefly, sample solution was centrifuged through an RNeasy column to bind the RNA to the column. Then, 10 µl of RNase-Free DNase (Qiagen) mixed with 70 µl of buffer RDD was added in the middle of each column for on-column DNase digestion which was carried out for 15 minutes at room temperature. The RNeasy column then was washed once with RW1 buffer (to wash membrane-bound RNA) and twice with RPE buffer (to remove traces of salts

remained on the column). The RNA was eluted using 50 µl of RNase-free water, and analysed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, USA).

2.5.1.2 RNA extraction from cells grown in tissue culture

CCL cells were grown in tissue culture as described previously (Section 2.4.2-2.4.3). In brief, cells cultured at passage 2-3 in 24-well plates were lysed by the addition of 1 ml Tri-reagent for 1 - 2 minutes. The extract from each well was collected in a 1.5 ml RNase-free tube (Eppendorf, Stevenage, UK) and stored at -80° C until RNA extraction was performed.

To every 1ml Tri-reagent, 200 µl of chloroform (Sigma-Aldrich, MO, USA) was added and the sample shaken and left at room temperature for five minutes. The sample was centrifuged again (20,817 x g, 15 minutes, 4 °C) and the clear, upper aqueous phase was carefully removed and transferred to a fresh tube. Total RNA was precipitated by addition of 0.5 ml of isopropanol (Sigma-Aldrich) followed by brief shaking and subsequent incubation at room temperature for five minutes. The precipitate was recovered by centrifugation (20,817 x g, 10 minutes, 4 °C) and the supernatant was discarded. The total RNA pellet was washed by adding 1 ml of 70% ethanol (Sigma-Aldrich) and following further centrifugation the supernatant was removed with the pellet being air dried by leaving the sample tubes open inside a fume hood. The extracted mRNA was dissolved in 20 µl of RNase-free water and measured with a Nanodrop 1000 spectrophotometer.

2.5.1.3 Synthesis of cDNA

Reverse transcription was performed according to the manufacturer's instructions. Briefly, 1 µg of random primers (Promega, Madison, USA) was added to the total RNA solution (1 µg) and heated for five minutes at 70°C. Following this 5 µl of 5x Reverse Transcription buffer (Promega), 2 µl of 10 mM dNTP mix (Bioline, London, UK), 1 µl of 200 U/µl reverse transcriptase enzyme (Promega) and RNase-free water were added making a total volume of 25 µl. cDNA was synthesized by incubating the mixture at 37°C for an hour and then heated at 95°C for five minutes to inactivate the reverse transcriptase enzyme. cDNA samples were stored at -20 °C until required.

2.5.1.4 Quantitative PCR (qPCR)

Each cDNA sample was added to a GoTaq qPCR Mastermix containing Syber Green (Promega) along with sequence-specific forward and reverse primers of interested genes; IL-6, MCP-1, TNF-α, AGC, COL1A2, MMP-13, adiponectin, leptin, and visfatin (synthesised by Eurogentec, Seraing, Belgium). Primer sequences are shown in Table 2.1 (Clements et al., 2008). The optimal concentration for each pair of primers was determined by performing an assay with final concentrations of forward and reverse primers at 300 nM or 600 nM using Syber Green. Validation experiment for each set of primers and probe was performed in the thesis of Ryan (2008) to confirm that the amplification efficiencies were adequate. The reaction efficiency was 90.7%, and the correlation coefficient was 0.98.

Samples were assayed in duplicate using a A7300 Real-Time PCR machine (Applied Biosystems, Warrington, UK) with the following thermal cycling parameters; 50 °C for two minutes (for the passive reference dye measurement), denaturation at 95°C for 10 minutes to activate Taq Polymerase (for activating the hot start Taq Polymerase) followed by 40 cycles of cDNA amplification (15 seconds at 95°C and

60°C for 1 minute). Based on previous literature, β-actin and ribosomal protein S13 (RPS-13) were the most appropriate housekeeping genes to use in normalising gene expressions of interested genes using the CT values (the cycle number at which a defined fluorescence threshold was reached) (Tew et al., 2014, Leal et al., 2015). The data generated from the real time PCR were then exported to Microsoft Excel to analysis using $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001). Dissociation curve analysis was also performed to determine that a single qPCR product was present at the end of the PCR reaction.

2.5.2 Enzyme-linked immunosorbent assays (ELISA) for cytokine and adipokine protein measurements

Concentrations of IL-6, TNF-α, adiponectin and leptin were determined in synovial fluid from both the clinical and control samples using commercially designed enzyme-linked immunosorbent assays (ELISA) kits. Undiluted synovial fluid was analysed for TNF-α (R&D Systems, Minneapolis, USA). Synovial fluid samples were diluted 1:3 with PBS to allow measurement of IL-6 (R&D Systems), adiponectin (Biovendor, Brno, Czech Republic) and leptin (Cloud-Clone Corp, Houston, USA). All assays were performed according to the manufacturer's instructions, and the absorbance was measured with a microplate reader (Multiskan EX, Thermo Scientific, Vantaa, Finland) at 450 nm with the correction wavelength set at 570 nm. Absorbance was converted to concentration using the standard curve derived from serially-diluted standards (Zhang et al., 2010, Conde et al., 2011, Kathrani et al., 2012, Jeon et al., 2015).

2.6 Statistical analysis

Shapiro-Wilk test was used for testing the normality of data. The differences in dog breed and neutering status between controls and cases were determined by Fisher's exact test. Weighted Cohen's Kappa was used to assess observe intra- and inter-observer agreements for radiographic scoring. The nonparametric Mann-Whitney U test was used to compare body fat percentage between male and female dogs. Kendall's tau test was used to identify associations between age, bodyweight, BCS, body fat percentage and clinical presentation of dogs with CCLD. Comparisons of age, bodyweight, BCS and genes expression between control subjects and CCLD subjects were made by the non-parametric Mann-Whitney U test. Associations amongst local and systemic adipokines, ligament degradation biomarkers, and synovial fluid adipokines and inflammatory markers were assessed with Kendall's tau test. The differences among *in vitro* experimental groups was determined using non-parametric Friedman test, followed by the Conover post-hoc test. The difference between control and CCLD groups was tested with 2-way non-parametric ANOVA. Statistical software (StatsDirect version 3 for Windows, StatsDirect Ltd., Cheshire, UK) was used for the analysis, and data were considered statistically significant at $P<0.05$.

Chapter 3 Association between obesity and clinical presentation in canine cranial cruciate ligament disease

3.1 Introduction

Cranial cruciate ligament disease (CCLD) is one of the most important causes of pelvic limb lameness in dogs (Bennett et al., 1988, Johnson et al., 1994). Evaluating the clinical presentation of dogs by assessing the degree of pelvic limb lameness, gait analysis and radiographic examination, disease severity can be determined and treatment plans formulated (Jerram and Walker, 2003). Lameness is visually assessed in veterinary practice by observing the dog at a walk and trot, then assigning a grade from 0 (sound) to 5 or 10 (continuous non-weight bearing) (Mathews et al., 2014). However, such visual gait assessment can be subjective and prone to observer bias (Quinn et al., 2007). Therefore, objective observations of gait analysis such as kinetic and kinematic evaluation are the gold standard method for determining pelvic limb lameness associated with CCLD (Conzemius and Evans, 2012). Force plate measurements such as peak vertical force (PVF), vertical impulse (VI), falling slope (FS), as well as asymmetry indices (AI) of these variables have been used as tools for objective lameness assessment (Fanchon and Grandjean, 2007, Oosterlinck et al., 2011). In addition, since CCLD often leads to meniscal tears and the development of osteoarthritis (OA), radiographic examination of the stifle joints is necessary to determine progression of degeneration of the joints and to plan an appropriate treatment for dogs with CCLD (Bennett and May, 1991, Vasseur and Berry, 1992).

Obesity is characterised by an expansion of excess white adipose tissue (WAT) mass that can lead to adverse health effects in humans (Cao, 2014). Obesity in dogs increases morbidity and mortality risk for various diseases including musculoskeletal disorders (Frye et al., 2016). Several techniques for measuring obesity in dogs such

as densitometry, dual-energy X-ray absorptiometry (DEXA), electrical impedance and morphometric methods have previously been described (German, 2006). Evaluating body condition scores (BCS), based on visual assessment and palpation, is most commonly used by veterinarians as it is inexpensive, convenient and non-invasive (Burkholder, 2000). Nonetheless, the assessment of BCS is subjective (German, 2006); therefore, DEXA is used as the gold standard for measuring body fat in many studies (Larsson et al., 2014, German et al., 2015).

Increased adiposity is a risk factor for lower limb injury in humans including unilateral anterior cruciate ligament (ACL) disease (Mattila et al., 2007, Takata et al., 2007). Studies have also highlighted the association between increase bodyweight and the development of canine CCLD (Whitehair et al., 1993, Witsberger et al., 2008). Compared with dogs in ideal bodyweight, obese dogs have 3.76 times the risk of developing CCLD (Adams et al., 2011). However, it remains unclear whether this increased risk is due to the increased bodyweight of dogs alone or also related to the effects of having excessive amounts of adipose tissue. Therefore, the main objective of this part of the study was to study the effects of both mechanical and humoral factors in dogs with CCLD with varying amounts of adipose tissue.

3.1.1 Aims

- To observe the association between both mechanical factor of obesity (bodyweight) and body condition score (BCS) with the clinical presentation (lameness score, gait variables from force plate measurements, radiographic examination score) in dogs with CCLD.

3.2 Methods

3.2.1 Patient selection and signalment

Dogs referred for surgical treatment of CCLD at Small Animal Teaching Hospital, University of Liverpool were recruited to the study from April 2014 to December 2015. Dogs were included in the study if they had a diagnosis unilateral CCLD as determined by presence of the cranial drawer sign, and were excluded if they had bilateral disease or obvious pain upon orthopaedic examination in other joints. Informed owner consent was obtained, according to local institutional ethical requirements (VREC192, see Section 2.1.2 in Chapter 2). Animal signalment including dog breed, sex, age and bodyweight were recorded as described previously (Chapter 2 Section 2.1.2).

3.2.2 Assessment of obesity and adiposity in dogs with CCLD

Measurements for determining obesity in the recruited animals were obtained as described in Chapter 2 (Sections 2.2 and 2.3.4). In brief, we used a nine-point scale BCS system to determine body composition of dogs (see Chapter 2 Section 2.2, Appendix 1). In addition, a DEXA scan was performed to precisely define the percentage of body fat mass (Chapter 2 Section 2.3.4).

3.2.3 Assessment of the clinical presentation of dogs with CCLD

The clinical presentation of CCLD dogs was assessed by determining lameness scores, force plate measurements and radiographic examination scores (Chapter 2 Section 2.3.1 – 2.3.3).

3.2.3.1 Lameness score

Lameness of CCLD dogs was visually evaluated during walking by an ECVS resident or RCVS or ECVS Diploma holding orthopaedic surgeon, and scored on a scale of 0 – 10, as no detectable lameness (grade 0), and most severe lameness (grade 10) as described in Chapter 2 Section 2.3.1.

3.2.3.2 Kinetic gait analysis using force plate measurements

Kinetic gait analysis using force plate measurements were performed at walk (velocity 1.0–1.3 m/s) prior to surgical treatment for CCLD. Data including peak vertical force (PVF), vertical impulse (VI), falling slope (FS) and asymmetry indices (AI) of these variables (AI PVF, AI VI, AI FS) of the affected limbs with at least 5 replicates were collected and analysed (see Chapter 2 Section 2.3.2).

3.2.3.3 Radiographic examination and scoring

Paired sets of anonymised radiographs (mediolateral and caudocranial projections) of the affected stifle joints (see Appendix 2) were scored by two investigators: WS (an MVM) and EC (a board-certified diplomate in veterinary surgery [DipECVS]). Each observer scored the radiographic images of each affected stifle joint independently twice (at least 2 weeks apart) for evidence of osteophytosis based on a 5-point Likert scale, with no osteophytosis (score 0), mild osteophytosis (score 1), moderate osteophytosis (score 2), marked osteophytosis (score 3), and severe osteophytosis (score 4) (Innes et al., 2004) (see Chapter 2 Section 2.3.3) .

3.2.4 Statistical analysis

Data were expressed as mean ± standard deviation (SD), median and range, unless where indicated. Weighted Cohen's Kappa was used to assess observe intra-

and inter-observer agreements for radiographic scores (described in Section 3.2.3.3); results between 0.01 and 0.20 were classed as slight agreement, those between 0.21 and 0.40 were classed as fair agreement, those between 0.41 and 0.60 were classed as moderate agreement, those between 0.61 and 0.80 were classed as substantial agreement, and those between 0.81 and 0.99 were classed as almost perfect agreement (Landis and Koch, 1977). The mean radiographic score from both observers was used in the final statistical analysis.

The nonparametric Mann-Whitney U test was used to compare body fat percentage derived from DEXA (Section 3.2.2) between male and female dogs. Since the data were not normally distributed, as determined by a Shapiro-Wilk test. Kendall's tau test was used to compare associations between body fat percentage and age, bodyweight and BCS. Finally, Kendall's tau was also used to determine the association of age, bodyweight and body fat with the clinical presentation of dogs with CCLD (Section 3.2.3). All data were analysed using statistical software (StatsDirect version 3 for Windows; StatsDirect Ltd., Cheshire, UK). Data were considered statistically significant at $P < 0.05$.

3.3 Results

3.3.1 Signalment and clinical presentation of dogs with CCLD

Thirty dogs with CCLD met the inclusion criteria for the study. Several breeds of dog were represented including mixed breed (3), Siberian Huskies (3), Staffordshire Bull Terriers (3), Border Collies (2), Golden Retrievers (2), Labrador Retrievers (2), Rottweilers (2), West Highland White Terriers (2), American Bulldog (1), Beagle (1), Border Terrier (1), Boxer (1), Bullmastiff (1), Cocker Spaniel (1), English Springer Spaniel (1), Flat Coated Retriever (1), German Shepherd dog (1), Labradoodle (1) and St Bernard (1).

There were entire female (3), neutered female (11), entire male (1) and neutered males (15) dogs recruited in this study. Median age was 62 months (range 18-126 months), whilst median bodyweight was 29.4 kg (range 9.8 – 64.1 kg).

3.3.1.1 Body condition score and body fat percentage

Median BCS and body fat percentage of dogs recruited in this study were score 6 out of 9 (range 3 – 8) and 35.0% (range 7.7 – 51.7%) respectively (Table 3.1).

Table 3.1 Body condition score and body fat of dogs with CCLD.

Body composition	Mean ± SD	Median	Range
Body condition score (0/9-9/9)	5.7 ± 1.2	6	3 - 8
Body fat (%)	32.0 ± 12.4	35.0	7.7 - 51.7

3.3.1.2 Lameness score and kinetic gait analysis

Median lameness score was 5/10 (range 2/10 – 10/10); the results for kinetic gait analysis parameters from the force platform are presented in Table 3.2.

Table 3.2 Values obtained from the measurement of lameness in dogs with CCLD using both subjective lameness scores and objective kinetic gait analysis.

Gait variable	Mean ± SD	Median	Range
Lameness score	5.8 ± 2.9	5	2 - 10
Gait parameters			
PVF (%BW)	36.2 ± 14.4	34.1	17.4 – 67.8
VI (%BW)	7.2 ± 3.0	6.7	2.0 – 15.9
FS (%BW/msecs)	-0.2 ± 0.2	-0.2	-0.6 – -0.1
AI PVF (%)	52.6 ± 33.8	51.1	2.1 – 134.5
AI VI (%)	54.4 ± 39.5	48.2	1.9 – 129.9
AI FS (%)	40.2 ± 38.4	29.2	0.6 – 166.7

PVF: peak vertical force; VI: vertical impulse; FS: falling slope; AI: asymmetry index; BW: bodyweight; msecs: milliseconds

3.3.1.3 Radiographic scoring

The median radiographic score of affected stifle joints following two blinded measurements by two investigators for CCLD dogs was 1 out of 4 (range 0/4 - 3/4). The intra-observer agreements from each investigator were substantial (Kappa 0.69 and 0.79 for WS and EC respectively, Table 3.4), but only fair agreement was obtained for inter-observer agreement (Kappa 0.28, Table 3.3).

Table 3. 3 Intra- and inter- observer agreements following blinded radiographic assessment of affected canine stifle joints.

| | Intra-observer agreement | | Inter-observer agreement |
	WS	EC	WS and EC
Kappa	0.69	0.79	0.28
95 %CI	0.53 -0.85	0.62 -0.96	0.12 - 0.45
P	< 0.001	< 0.001	0.002

WS: Wipawee Saengsoi; EC: Eithne Comerford; CI: confidence interval

3.3.2 Associations between body fat composition and animal signalment

Figures 3.1 represents the scatter plots along with regression trend of associations between body fat percentage and animal signalment. There were no differences in body fat percentage between male and female dogs with CCLD (P = 0.984, Figure 3.1A). However, body fat percentage was positively associated with age (Kendall's tau = 0.30, P = 0.019; Figure 3.1B), bodyweight (Kendall's tau = 0.28, P = 0.029; Figure 3.1C) and BCS (Kendall's tau = 0.62, P < 0.001; Figure 3.1D).

3.3.3 Relationship between all parameters related to obesity and the clinical presentation of dogs with CCLD

The association between factors-related to obesity, as observed in Section 3.3.2 (age, bodyweight and body fat), and clinical presentation of CCLD dogs (lameness score, radiographic score, force platform data) were determined. A weak negative correlation was found between bodyweight and PVF (Kendall's tau = -0.31, P = 0.016), whereas a positive correlation between bodyweight and FS was found (Kendall's tau = 0.36, P = 0.006, Table 3.4). However, no associations were found between age and body fat composition with any other clinical parameters (Table 3.4)
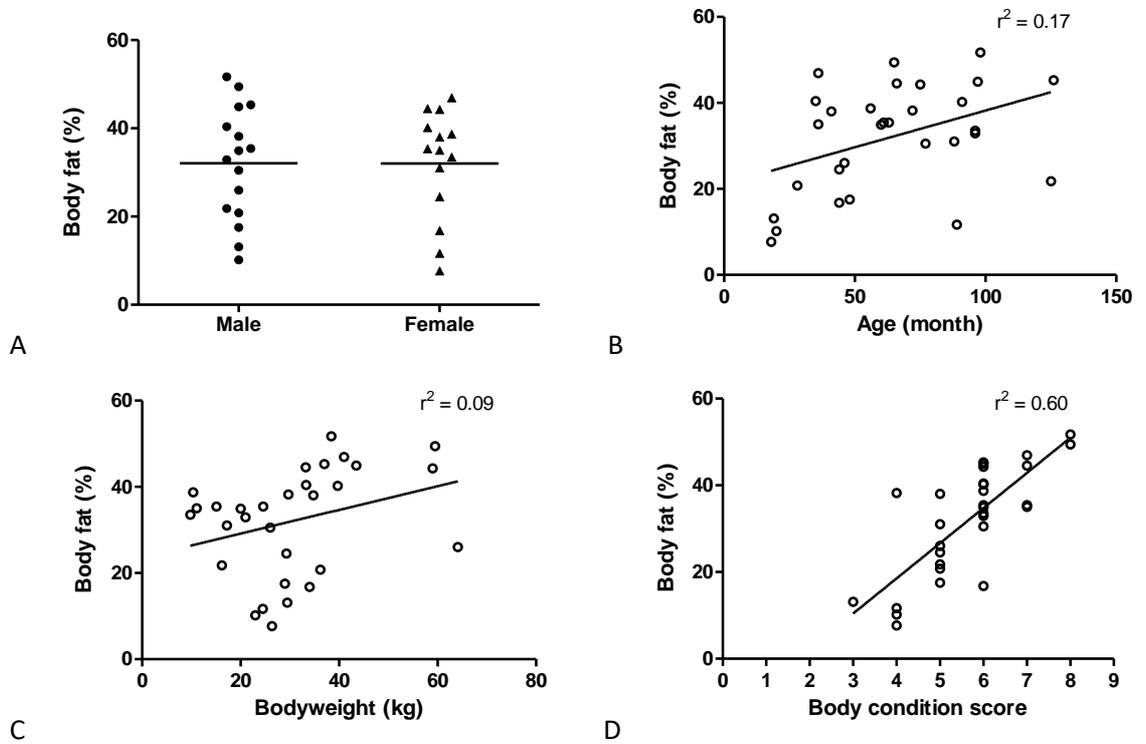
Figure 3.1 Graphical presentation of the associations between percentage body fat mass and animal signalment.

Scatter plots and regression trend of comparison between body fat percentage with sex (A), age (B), bodyweight (C) and body condition score (D) in dogs with CCLD. kg: kilogram. The scatter plots show no difference of body fat percentage between male and female dogs, the horizontal lines represent mean (A). Whilst there were positive correlations between body fat percentage with age (B), bodyweight (C) and body condition score (D) of dogs.

Table 3.4 Relationship of all parameters used to determine obesity and the clinical presentation of dogs with CCLD.

		Lameness score	PVF	VI	FS	AIPVF	AIVI	AIFS	Radiographic score
Age	Kendal	0.13	0.12	0.16	-0.03	-0.11	-0.15	0.08	0.09
	P	0.352	0.353	0.211	0.830	0.412	0.253	0.544	0.554
Bodyweight	Kendal	0.03	-0.31*	0.09	0.36*	0.06	-0.01	-0.13	0.06
	P	0.832	0.016	0.464	0.006	0.630	0.929	0.309	0.684
Body fat %	Kendal	0.06	-0.11	-0.12	0.06	0.01	0.11	-0.06	0.22
	P	0.688	0.392	0.353	0.643	0.915	0.392	0.643	0.137

PVF: peak vertical force; VI: vertical impulse; FS: falling slope; AI: asymmetry index (*P < 0.05)

3.4 Discussion

Increased body fat mass has been found in humans with chronic unilateral ACL disease (Takata et al., 2007). Bodyweight and body mass index (BMI) are also risk factors for non-contact ACL injury in women (Uhorchak et al., 2003). In dogs, obesity increases the risk of CCLD by 4 times (Adams et al., 2011). Therefore, the hypothesis for this study was that both mechanical and humoral factors of obesity could be involved in pathophysiology of CCLD. Specifically, this study aimed to investigate the link between obesity and the clinical presentation of canine CCLD to elucidate any significant associations, which could be used in the future management of this condition.

3.4.1 Signalment, bodyweight, and body fat mass

Intrinsic factors of dogs such as breed, sex, neuter status, age and bodyweight have been identified as possible risk factors for CCLD (Whitehair et al., 1993, Witsberger et al., 2008). Although CCL injury can be seen in all breeds of dog (Jerram and Walker, 2003), it occurs more commonly in those from large breeds (Witsberger et al., 2008). Almost half of dogs recruited in this study were large- or giant-dog breeds; however, some small and medium dog breeds were also included. The number of male and female dogs represented was approximately the same. No sex differences for CCLD have been reported (Duval et al., 1999, Witsberger et al., 2008), besides one recent study which reported twice the risk of CCLD in female dogs as compare to the males (Adams et al., 2011). A further finding of this study was that the majority of dogs were neutered. In a similar manner, previous studies have demonstrated that neutered male and female dogs are 1.68 - 2.35 times more likely to have CCLD than entire dogs (Witsberger et al., 2008). However, it has been suggested that owners of neutered dogs are more likely to bring their dogs to veterinarians than owners of sexually intact dogs (Witsberger et al., 2008). In the current study, median age of dogs with CCLD was five years, a finding that is consistent with previous work showing dogs older than 4 years old and large to giant

breeds are reportedly predisposed to CCLD (Whitehair et al., 1993). We observed a median bodyweight of 29.4 kg in dogs with CCLD in our cohort. In addition, most dogs in our study were considered overweight with a BCS of at least 6 out of 9.

To the best of our knowledge, this is the first study that has attempted to determine the relationship between body fat percentage (determined by DEXA) and canine CCLD. DEXA was used because it is known to be precise and accurate (German et al., 2010a). Indeed, a statistically significant association between BCS and body fat percentage was identified, whilst a moderate correlation between bodyweight and body fat percentage was also seen. In addition to these findings, a positive correlation between dog age and body fat percentage was noted. In a similar manner, a previous study has documented that as the age of dogs increases, so too does the risk of being overweight, as measured by BCS (Colliard et al., 2006).

3.4.2 Relationship of obesity and the clinical presentation of dogs with CCLD

3.4.2.1 Visual lameness

Pelvic limb lameness is commonly seen in dogs with CCLD where the severity can be varied (Jerram and Walker, 2003). A previous human study has demonstrated that the risk of osteoarthritis (OA) can be related to both adipose mass and central adiposity (Wang et al., 2009), whilst reducing body fat is more important than reducing in bodyweight for symptomatic relief of knee OA in humans (Toda et al., 1998). However, no statistically significant correlations were noted between bodyweight or body fat percentage with visual lameness of the affected dogs in this study. It is generally known that lameness evaluation can be challenging in dogs, and there is evidence of low inter-rater agreement when visually judging lameness (Waxman et al., 2008).

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3.4.2.2 Force plate-detected lameness

It has been reported that subjective scoring scales poorly agree with objective lameness assessments such as force plate measurement (Quinn et al., 2007). Kinetic gait analysis using a force platform represents the gold standard method of objectively assessing lameness in dogs (Conzemius and Evans, 2012). Associations were identified between bodyweight and certain gait variables measured with force plate (PVF and FS) in this study. The PVF is the most accurate measurement for kinetic lameness evaluation and usually correlates with vertical impulse (Fanchon and Grandjean, 2007). In dogs with CCLD, PVF of the affected limb is usually reduced (Conzemius et al., 2005, Fischer et al., 2013). The negative correlation between bodyweight and PVF found in our study might imply that dogs with greater bodyweight are less willing to weight bear on the lame limb during the gait cycle. Therefore, a low magnitude of ground reaction force was observed (Fischer et al., 2013). The FS value indicates the time for a dog to unload its limb once maximum force has been reached; the negative results seen in dogs with CCLD, compared with control dogs, suggest more rapid unloading possibly as a the result of pain (Evans et al., 2005). Negative FS results have been observed when dogs with hip OA are treated with placebo comparing to non-steroidal anti-inflammatory drugs (Malek et al., 2012). However, the author of that study interpreted the changes in FS value detected in placebo-treated dogs as an undesirable effect. The positive correlation between bodyweight and FS observed in this study indicated that heavier dogs took a longer time to unload their injured limb. Likewise, previous studies in obese children have shown that the duration of the gait cycle is longer in obese subjects than normal-weight subjects at both slow and fast walking speeds. It was speculated in this study that they spend more time in stance phase and double support phase in which bodyweight is supported by both legs (Hills and Parker, 1992, McGraw et al., 2000). This might suggest a possible link between movement inefficiency in the gait cycle of dogs with a bigger bodyweight.

3.4.2.3 Radiographic score

Canine CCLD is a leading cause of stifle OA (Vasseur and Berry, 1992) and radiographs are routinely performed to detect progression of OA occurring in affected stifles (Jerram and Walker, 2003). Osteophytes can occur at the margins of the femoral trochlea as early as three days after CCLD using Pond Nuki model (Gilbertson, 1975). Osteophyte score is correlated with OA progression secondary to CCLD (Innes and Barr, 1998). Therefore, a grading system of radiographic OA, based on osteophyte formation in canine stifles joints has been developed and validated (Innes et al., 2004). The scoring system used in this study showed substantial intra-observer agreement as was previously reported (Innes et al., 2004). However, the inter-observer agreement was fair (0.28). This result may well have been the result of differences in experiences between two observers. Although evaluating osteophytes as part of a scoring system is convenient and give a good intra-observer agreement, there are also other scoring systems that might be more sensitive for screening OA progression (Vasseur and Berry, 1992, Au et al., 2010).

In previous research, more osteophyte formation is known to occur in larger and more obese dogs, although no significant correlation between bodyweight and osteophyte formation was found in those studies (Heffron and Campbell, 1979, Rayward et al., 2004). Our study observed that different grades of radiographic OA occurred in the affected stifle joints; however, there were no statistically significant associations of radiographic score with body fat percentage, bodyweight or age of dogs.

3.4.3 Effect of mechanical factors of obesity on CCL

Previous studies on cyclic mechanical stretching of tendon fibroblasts suggest that increased inflammatory mediator production can lead to tendinitis (Wang et al., 2003), a phenomenon that might also occur in ligament cells. Furthermore, a

histological study has demonstrated alterations of the cellular and extracellular matrix in torn CCL, which have resulted from the cumulative effects of remodelling and adaptation to mechanical loading (Hayashi et al., 2003). These findings support the theory that obesity can contribute to the incidence of CCLD in large size and heavy weight dogs, as higher loads would be placed on the stifles (Griffon, 2010). The mechanism could involve accumulative effect of repetitive loading resulting in accelerating degenerative processes, as well as weakening of the ligament and surrounding soft tissue fatigue which can could contribute to ligament inflammation and degeneration (Johnson and Johnson, 1993, Moore and Read, 1996a).

The most notable limitation of this study was the fact that it was small yet there was a large variety of dog breeds, sizes, and degrees of body fat. Given such variability, the study was likely to be somewhat underpowered therefore reducing obesity factors such as studying differences between breeds, a limited range of bodyweights and age, as well as recruiting more subjects should be considered in future investigations.

3.5 Conclusion

This study investigated the effect of obesity, in terms of bodyweight and body fat percentage, on clinical presentation of dogs with CCLD, including lameness scores, gait parameters from force plate analysis and radiographic scores. Some association was seen between bodyweight and the clinical manifestation of CCLD, in terms of the severity of lameness as detected by force plate. In contrast, there was no significant association with body fat mass. Further studies are required to determine the basis for the association between bodyweight and CCLD severity.

Chapter 4 Adipokines as metabolic mediators in canine cranial cruciate ligament disease

4.1 Introduction

Cranial cruciate ligament disease (CCLD) in dogs is a degenerative condition with a complex aetiopathogenesis involving inflammatory stifle arthritis, collagen degradation (Muir et al., 2005, Doom et al., 2008) and many predisposing factors including obesity (Taylor-Brown et al., 2015). White adipose tissue has many functions and is now considered to be an endocrine organ producing a variety of hormones, termed adipokines. The most well-studied adipokines include leptin, adiponectin, and visfatin (Lagowska and Jeszka, 2011). Other adipokines include cytokines and chemokines (sometimes referred to as adipocytokines) and these include interleukin-6 (IL-6), macrophage chemotactic protein-1 (MCP-1) and tumour necrosis factor-α (TNF-α) (Fain et al., 2004). Dysregulation of these adipokines can have local or systemic effects on inflammatory responses, which contribute to many obesity-associated disorders (Cao, 2014). In this respect, associations have been demonstrated between obesity-related metabolic factors and musculoskeletal disorders such as osteoarthritis (OA) (Grotle et al., 2008, Griffin et al., 2010). There is also *in vitro* evidence that adipokines, such as leptin and visfatin, can disrupt cartilage homeostasis and induce pro-inflammatory mediators such as IL-6 and IL-8 (Wang et al., 2015).

The infrapatellar fat pad (IPFP) is an active adipose tissue located in the knee joint and known to secrete various adipokines into the joint (Ioan-Facsinay and Kloppenburg, 2013). Previous studies have indicated a crosstalk between IPFP and other joint tissues such as cartilage and chondrocytes, which implies that this crosstalk might contribute to the pathogenesis of OA (Bastiaansen-Jenniskens et al., 2012, Bastiaansen-Jenniskens et al., 2013, Chen et al., 2016). In contrast, medium conditioned by OA IPFP have been found to have inhibitory catabolic effect on

articular cartilage (Bastiaansen-Jenniskens et al., 2012, Bastiaansen-Jenniskens et al., 2013, Chen et al., 2016).

Despite such research, the mechanisms by which obesity predisposes to OA remain unclear, not least whether there is an association between the systemic or local release of adipokines (Kwon and Pessin, 2013). Furthermore, there have been no studies assessing the association between obesity and CCLD. Therefore, our hypothesis is that chemical mediators secreted from either systemic or local (intra-articular stifle joint) adipose tissues may influence on CCLD pathophysiology and clinical presentation of dogs with CCLD.

4.1.1 Aims

- To examine differences in gene expression of pro-inflammatory cytokines, tissue degradation biomarkers, and adipokine expression in dogs with and without CCLD.
- To determine the association between both local and systemic adipokines, pro-inflammatory cytokines and tissue degradation biomarkers in CCL tissues and synovial fluid of dogs with CCLD.
- To investigate the relationship between chemical mediators in the stifle joint and the clinical presentation of dogs with CCLD.
- To examine the relationship of body fat composition and chemical mediators from both local and systemic adipose tissues of dogs with CCLD.

4.2 Methods

4.2.1 Animals and sample collection

Control group: Dog cadavers euthanased for reasons other than musculoskeletal disease were used as controls. Tissues including CCL and IPFP were collected from the right stifle joint and subcutaneous (SC) fat were collected from the right inguinal area of the cadavers (Chapter 2 Section 2.1.1).

Clinical group: Client-owned dogs referred for surgical treatment of CCLD were recruited. Dogs were included in the study if they had a diagnosis of unilateral CCLD as determined by presence of the cranial drawer sign and were excluded if they had bilateral disease or obvious pain upon orthopaedic examination in other joints. Ruptured CCL, IPFP tissues and synovial fluid were collected from the affected joint. SC tissues were obtained from the surgical site (Chapter 2 Section 2.1.2).

Animal signalment including breed, sex, neuter status, age, bodyweight and body condition score (see Appendix 1) of all dogs were measured and recorded. Ethical permission for using the control and clinical material was approved by the Veterinary Research Ethics Committee, School of Veterinary Science (VREC213 and VREC192) and signed owner consent was obtained for the clinical group (Chapter 2 Section 2.1).

4.2.2 Measurement of pro-inflammatory cytokines, tissue degradation biomarkers and adipokines gene expression in dogs with and without CCLD

Gene expression of pro-inflammatory cytokines, tissue degradation biomarkers and adipokines was measured in tissue samples as described in Chapter 2 Section 2.5. Briefly, RNA was extracted from ground tissues using guanidinium thiocyanate-phenol-chloroform extraction technique and was purified using RNeasy mini kit®. Reverse transcription was performed to synthesise cDNA, which was used in quantitative PCR (qPCR) assays. The assays were to determine expression for a

range of genes depending on the tissue type. IL-6, TNF-α, MCP-1, aggrecan (AGC), collagen-1 (COL-1) and matrix metalloproteinase (MMP)-13 were measured in CCLs. Adiponectin, leptin, visfatin, IL-6, TNF-α, MCP-1 and MMP-13 were measured in IPFP and SC.

4.2.3 Measurement of adipokines and pro-inflammatory cytokines in synovial fluid

Synovial fluid was obtained from dogs with CCLD for measurement of adipokine (adiponectin, leptin) and pro-inflammatory cytokine (IL-6, TNF-α) concentrations using ELISA commercial kits (TNF-α (R&D Systems); IL-6 (R&D Systems), adiponectin (Biovendor); leptin (Cloud-Clone Corp)). Briefly, 100 µl of diluted or undiluted synovial fluid were added to pre-coated wells in duplicate. All assays were performed according to the manufacturers' instructions and the absorbance was measured with a microplate reader at 450 nm with the correction wavelength set at 570 nm. Protein concentrations were calculated using the standard curve derived from the results of a set of serially-diluted standards (Chapter 2 Section 2.5.2).

4.2.4 Clinical presentation of dogs with CCLD

The clinical presentation of dogs with CCLD was assessed by lameness score, force plate measurement data including peak vertical force (PVF), vertical impulse (VI), falling slope (FS), together with asymmetry indices of these values and radiographic score.

4.2.4.1 Lameness score

Lameness of CCLD dogs was visual evaluated during walking, and scored on a Likert scale of 0 – 10, as no detectable lameness (grade 0), and most severe lameness (grade 10) (Chapter 2 Section 2.3.1).

4.2.4.2 Force plate measurement

Gait analysis using force plate measurement was performed at the walk (velocity 1–1.3m/s). Data including PVF, VI, FS and AI of these variables (AI PVF, AI VI, AI FS) were collected (Chapter 2 Section 2.3.2).

4.2.4.3 Radiographic score

Thirty paired sets of stifle radiographs (mediolateral and caudocranial projections) of the affected joints were scored twice by two blinded investigators: WS (DVM) and EC (DipECVS). Radiographic scores were assessed by the evidence of osteophytosis (see Appendix 2) based on a 5-point Likert scale, with no osteophytosis (score 0), mild (score 1), moderate (score 2), marked (score 3), and severe (score 4) (Innes et al., 2004). The average values from four rounds of scoring were used in data analysis (Chapter 2 Section 2.3.3).

4.2.5 Measurement of body composition

As part of the clinical investigations performed in dogs with CCLD prior to surgical management of this condition, the body fat composition was measured using fan-beam dual-energy X-ray absorptiometry (DEXA, Lunar Prodigy Advance; GE Lunar), and then analysed using enCORE 2004 software (v8.70.005, GE Lunar) (Chapter 2 Section 2.3.4).

4.2.6 Statistical analysis

Statistical software (StatsDirect version 3 for Windows, StatsDirect Ltd., Cheshire, UK) was used for statistical analysis. Data were expressed as median and range, unless indicated. The differences in dog breed and neutering status between controls and cases were determined by Fisher's exact test. Since the samples were not normally distributed, comparisons of age, bodyweight, BCS and genes expression between control subjects and CCLD subjects were made by the non-parametric Mann-Whitney U test. Associations amongst local and systemic adipokine profiles, ligament degradation biomarkers, and synovial fluid adipokines and inflammatory

markers were assessed with Kendall's tau test. A P value <0.05 was considered to be statistically significant.

4.3 Results

4.3.1 Baseline data of animal signalment

The control group comprised 26 dogs, from several breeds including Staffordshire Bull Terrier (20), mixed breed (3), Rottweiler (2), and Jack Russell Terrier (1). Seven of the dogs were entire female, 17 of the dogs were entire male and the remaining 2 were neutered male. There were 28 dogs in the CCLD group and the breeds included: mixed breed (3), Siberian Husky (3), Border Collie (2), Golden Retriever (2), Labrador Retriever (2), Rottweiler (2), Staffordshire Bull Terrier (2), West Highland White Terrier (2), American Bulldog (1), Beagle (1), Boxer (1), Bullmastiff (1), Cocker Spaniel (1), English Springer Spaniel (1), Flat Coated Retriever (1), German Shepherd Dog (1), Labradoodle (1), and St Bernard (1). There were entire female dogs (2), neutered female (10), entire male (1) and neutered male (15).

Dogs from retriever breeds (P = 0.024) and neutered dogs (P <0.001) were over-represented in the CCLD group compared with the control group (Table 4.1). Conversely, there were no differences in the disease occurrence between male and female dogs (P = 0.264, Table 4.1). In addition, age, bodyweight, and body condition score were all significantly greater in the dogs with CCLD than in the controls (P = 0.002, P = 0.004, and P < 0.001 respectively, Table 4.1). Median body fat was 35% (range 8 - 52%) in the CCLD dogs, but not measured in the control group given the fact that they were cadavers.

Table 4.1 Comparing baseline data of control and dogs with CCLD.

Category	Control (n=26)			Case (n=28)			P value
	Mean±SD	Median	Range	Mean±SD	Median	Range	
Being Retriever breed (n)	n = 0			n = 6			0.024
Being male (n)	n = 19			n = 16			0.264
Being neutered (n)	n = 2			n = 25			<0.001
Age (mt)	40.2±17.0	36	24 -96	63.7±29.9	62	18 -126	0.002
Body weight (kg)	22.1±5.9	22.8	11 -35	30.9±13.6	29.1	10.4 – 64.1	0.004
Body condition score (0/9-9/9)	4.2±1.0	4	2 -6	5.6±1.2	6	3 -8	<0.001

n: number of dogs; mt: month; kg: kilogram

4.3.2 Gene expression of chemical mediators in CCL and adipose tissue of control dogs and dogs with CCLD

Gene expression of pro-inflammatory cytokines (IL-6, MCP-1, TNF-α) and tissue degradation biomarkers (AGC, COL-1, MMP-13) were significantly greater in the CCL of dogs with CCLD compared to those in the controls (P = 0.006 for MCP-1 and P<0.001 for the other genes, Figure 4.1A). In contrast, within the IPFP, gene expression of both adiponectin and leptin were significantly greater in the control group (P = 0.001 and P = 0.029 respectively), but no difference in visfatin gene expression was found (P = 0.099, Figure 4.1B). Gene expression of IL-6 in IPFP derived from CCLD dogs was significantly greater (P = 0.006), whereas gene expression of MMP-13 was markedly lower than the control group (P = 0.007, Figure 4.1B). However, no difference in the gene expression of MCP-1 and TNF-α was found between these two groups, with P values of 0.654 and 0.539 respectively (Figure 4.1B). Finally, in SC adipose tissue, gene expression of leptin (P < 0.001), IL-6 (P = 0.048), MCP-1 (P = 0.001), TNF-α (P = 0.001) and MMP-13 (P = 0.009) were greater in dogs with CCLD than in control dogs (Figure 4.1C).
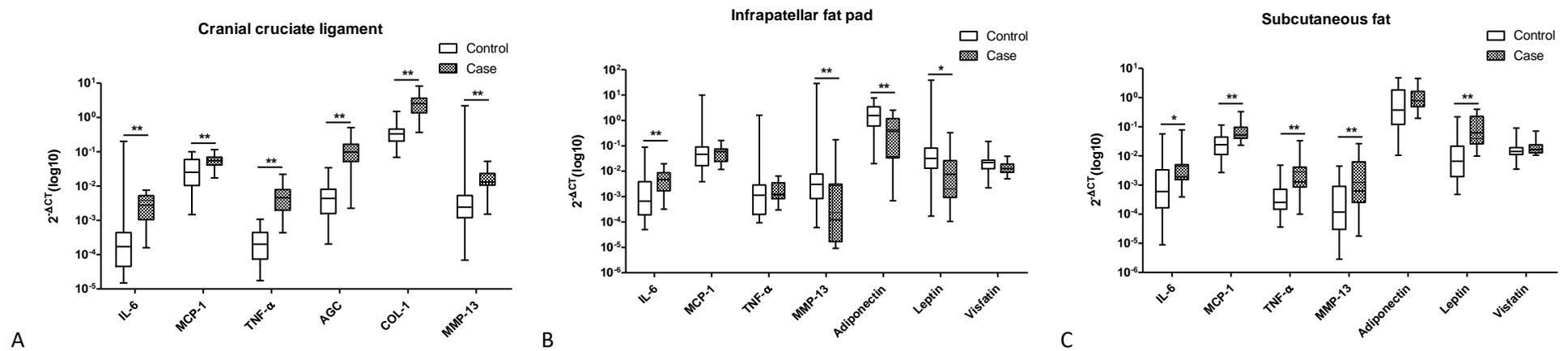
Figure 4.1 Comparing genes expression of chemical biomarkers in CCLs and adipose tissues between control dogs and dogs with CCLD.

The differences in genes expression of adipokines and chemical biomarkers in cranial cruciate ligaments (A), infrapatellar fat (B) and subcutaneous fat (C) are shown between controls and cases. Boxes contain values from 1st to 3rd quartile, lines inside boxes indicate median values and endpoints of vertical lines represent maximum and minimum (*P < 0.05, **P < 0.01). IL-6: interleukin-6; MCP-1: macrophage chemotactic protein-1; TNF-α: tumour necrosis factor-α; AGC: aggrecan, COL-1: type I collagen; MMP-13: matrix metalloproteinase-13. Figures show all adipokines and chemical biomarkers were significantly increased in CCLs of dogs with CCLD (A). Adiponectin, leptin and MMP-13 were significantly decreased, whereas IL-6 was significantly increased in infrapatellar fat of the CCLD group (B). There was a significant increase in leptin and all chemical biomarkers in subcutaneous fat of the CCLD group (C).

4.3.3 The association of local and systemic adipokines with CCLD biomarkers in synovial fluid and CCL tissues

Protein level of adipokines and pro-inflammatory cytokines in synovial fluid of dogs with CCLD measured by ELISA are shown in Table 4.2. Focusing on two sources of adipose-derived adipokines of dogs with CCLD, there were some associations between adipokines in IPFP and SC and the disease markers in synovial fluid and CCL tissues. In IPFP, adiponectin and visfatin gene expression were positively correlated with both TNF-α concentration in synovial fluid (P = 0.045) and its gene expression in CCL (P = 0.033), whilst leptin was inversely association with MMP-13 in CCLs (P = 0.042, Table 4.3). Conversely, although the expression of visfatin in SC adipose tissue was positively correlated with TNF-α concentration in synovial fluid, it was negatively correlated with TNF-α gene expression in CCL tissues (P = 0.045 and P = 0.046 respectively, Table 4.3).

Table 4.2 Protein level of adipokines and pro-inflammatory cytokines in synovial fluid of dogs with CCLD.

	Mean±SD	Median	Range
Adiponectin (ng/ml)	57.2±23.2	49.0	35.9 - 114.7
Leptin (ng/ml)	16.9±16.5	8.9	2.1 - 52.2
IL-6 (pg/ml)	614.7±542.3	450.6	29.5 - 1,848.6
TNF-α (ng/ml)	4.0±6.3	0.9	0.9 – 18.9

Table 4.3 Association between gene expression level of local and systemic adipokines with protein level of pro‑inflammatory cytokines in synovial fluid and gene expression level of tissue degradation biomarkers in cranial cruciate ligaments.

Adipokines		Synovial fluid		Cranial cruciate ligament					
		IL-6	TNF-α	IL-6	MCP-1	TNF-α	AGC	COL-1	MMP-13
Infrapatellar fat									
Adiponectin	Kendal	0.08	0.49*	-0.02	0.03	0.06	0.15	0.33	-0.16
	P	0.731	0.045	0.928	0.869	0.742	0.384	0.053	0.365
Leptin	Kendal	0.00	0.34	0.03	-0.02	-0.01	0.15	0.30	-0.39*
	P	1.000	0.193	0.870	0.928	0.961	0.418	0.105	0.042
Visfatin	Kendal	0.19	-0.25	0.23	0.17	0.32*	0.03	-0.10	-0.03
	P	0.340	0.266	0.151	0.278	0.033	0.849	0.505	0.830
Subcutaneous fat									
Adiponectin	Kendal	0.04	-0.39	0.29	0.03	-0.02	0.19	0.04	0.06
	P	0.857	0.133	0.088	0.861	0.897	0.227	0.809	0.697
Leptin	Kendal	-0.06	-0.25	0.25	0.02	-0.08	0.14	-0.09	0.12
	P	0.835	0.365	0.177	0.934	0.622	0.421	0.600	0.472
Visfatin	Kendal	0.33	0.55*	0.03	-0.01	-0.33*	-0.18	0.24	-0.15
	P	0.211	0.045	0.869	0.970	0.046	0.270	0.136	0.382

IL-6: interleukin-6; TNF-α: Tumour necrosis factor-α; MCP-1: macrophage chemotactic protein-1; AGC: aggrecan; COL-1: type 1 collagen; MMP-13: matrix metalloproteinase-13 (*P <0.05).

4.3.4 The association between chemical mediators in the stifle joint and the clinical presentation in dogs with CCLD

There was an association between leptin concentration in synovial fluid and lameness of dogs with CCLD. Leptin was positively correlated with lameness score (visual gait assessment, P = 0.032), whilst leptin was negatively correlated with PVF from the force plate measurement (P = 0.015, Table 4.4). However, there was no association between chemical mediators expressed in CCL tissues and the clinical presentation of lame dogs (Table 4.4).

Table 4.4 Association between protein level of pro-inflammatory cytokines in synovial fluid, gene expression level of tissue degradation biomarkers in cranial cruciate ligaments and clinical presentation of dogs with CCLD.

		Lameness score	PVF	VI	FS	AI PVF	AI VI	AI FS	Radiographic score
Synovial fluid									
Adiponectin	Kendal	-0.22	0.20	-0.12	-0.24	0.18	0.22	0.12	-0.28
	P	0.290	0.299	0.520	0.216	0.347	0.255	0.520	0.182
Leptin	Kendal	0.52*	-0.51*	-0.23	0.23	0.10	0.26	0.03	0.13
	P	0.032	0.015	0.272	0.272	0.625	0.222	0.903	0.573
IL-6	Kendal	-0.15	-0.16	-0.16	0.02	0.10	0.25	-0.12	-0.36
	P	0.492	0.426	0.426	0.921	0.619	0.196	0.551	0.099
TNF-α	Kendal	-0.15	0.09	0.12	0.00	0.00	0.03	0.00	-0.24
	P	0.553	0.678	0.580	1.000	1.000	0.890	1.000	0.330
Cranial cruciate ligament									
IL-6	Kendal	0.18	0.02	0.04	0.12	-0.15	-0.19	0.01	0.33
	P	0.302	0.888	0.800	0.446	0.324	0.225	0.933	0.064
MCP-1	Kendal	0.19	-0.04	-0.15	-0.00	-0.03	0.07	-0.03	0.09
	P	0.287	0.800	0.324	0.978	0.844	0.632	0.844	0.613
TNF-α	Kendal	-0.15	0.02	0.02	-0.01	-0.02	-0.02	0.05	0.08
	P	0.357	0.882	0.882	0.960	0.882	0.882	0.728	0.652
AGC	Kendal	0.04	-0.08	-0.16	-0.08	0.19	0.21	0.15	-0.25
	P	0.797	0.575	0.262	0.575	0.191	0.135	0.283	0.128
COL-1	Kendal	0.05	-0.09	-0.09	0.05	0.06	0.15	-0.04	-0.18
	P	0.753	0.544	0.544	0.744	0.674	.283	0.779	0.289
MMP-13	Kendal	0.06	-0.06	-0.04	-0.04	0.14	0.08	0.00	-0.21
	P	0.716	0.692	0.771	0.812	0.355	0.616	0.979	0.223

PVF: peak vertical force; VI: vertical impulse; FS: falling slope; AI: asymmetry index; IL-6: interleukin-6; TNF-α: tumour necrosis factor-α; MCP-1: macrophage chemotactic protein-1; AGC: aggrecan; COL-1: type 1 collagen; MMP-13: matrix metalloproteinase-13 (*P <0.05).

4.3.5 The relationship between body fat and chemical mediators in local and systemic adipose tissues

In SC fat of dogs with CCLD, body fat mass (measured by DEXA) was positively correlated with adiponectin (P = 0.004) and leptin (P = 0.025) gene expression, but negatively correlated with MMP-13 (P = 0.001) gene expression. However, none of the mRNAs measured in the infrapatellar fat pad was associated with body fat mass (P > 0.05, Table 4.5).

Table 4.5 Correlation between regional body fat and chemical mediators in adipose tissues.

		Infrapatellar fat							Subcutaneous fat						
		Adiponectin	Leptin	Visfatin	IL-6	MCP-1	TNF-α	MMP-13	Adiponectin	Leptin	Visfatin	IL-6	MCP-1	TNF-α	MMP-13
Body fat	Kendal	-0.22	-0.15	0.22	0.12	0.23	0.13	0.09	0.44**	0.36*	-0.20	0.26	-0.36	-0.15	-0.55**
	P	0.173	0.410	0.103	0.372	0.082	0.361	0.509	0.004	0.025	0.215	0.123	0.100	0.418	0.001

IL-6: interleukin-6; MCP-1: macrophage chemotactic protein-1; TNF-α: tumour necrosis factor-α; MMP-13: matrix metalloproteinase-13 ($*P <0.05$, $**P < 0.01$).

4.4 Discussion

4.4.1 Differences in gene expression associated with CCLD

4.4.1.1 Differences in gene expression of pro-inflammatory cytokines and tissue degradation biomarkers in disease and non-disease CCLs

Several studies in humans have demonstrated that anterior cruciate ligament (ACL) injury induces increased inflammatory cytokine (IL-6, MCP-1 and TNF- α) expression, proteolysis of aggrecan, type I and type II collagen concentrations in synovial fluid, shortly after injury (Irie et al., 2003, Cuellar et al., 2010, Struglics et al., 2015). Gene expression of cytokines and tissue degradation biomarkers such as IL-6, TGF-β1, collagen type 1 and MMPs have also been observed in different stages of human injured ACL tissues (Naraoka et al., 2012). However, few studies have investigated differences in gene expression between normal and diseased CCLs in any species (Clements et al., 2008, Leal et al., 2015). Results from this study demonstrated increased expression of IL-6, TNF-α and MCP-1 mRNA in injured CCLs when compared with controls. These results are consistent with previous studies which have demonstrated increased protein concentrations of IL-6 and TNF-α in synovial fluid from canine OA joints secondary to CCLD compared to controls (Hay et al., 1997, Fujita et al., 2006).

Extracellular matrix (ECM) turnover is a normal physiological process in healthy tissue which is altered in the diseased state (Stetler-Stevenson, 1996); for example ECM turnover is more dynamic in injured ligament tissue, suggesting an attempt at tissue reparation (Lo et al., 1998, Comerford et al., 2004). The results of the current study are comparable to a previous canine study that demonstrated up-regulation of mRNA expression for the genes involved in matrix remodelling, such as collagen type I and aggrecan, in injured ligaments compared to normal CCLs (Clements et al., 2008). Consistent with this study, the expression of MMP-13 is upregulated in human ruptured ACL (Muir et al., 2005), and also in canine and rabbit ruptured CCL tissues (Muir et al., 2005, Clements et al., 2006, Attia et al., 2010).

MMP-13 is one of the essential mediators playing roles in collagen destruction and degenerative joint diseases such as OA (Leeman et al., 2002, Wang et al., 2013). Taken together, expression of these biomarkers observed in the injured CCLs suggests changes in the inflammation, remodelling, and degradation processes occurring in these tissues.

4.4.1.2 Differences in gene expression of pro-inflammatory cytokines and adipokines in disease and non-disease infrapatellar fat

Human studies have identified IPFP as a local source of adipose tissue located within the joint capsule of the knee, close to the articular cartilage, synovium and bone (Saddik et al., 2004). Recently, IPFP has been suggested to be a source of local inflammatory cytokines and adipokines, which may contribute to the OA disease process (Distel et al., 2009, Iwata et al., 2013). There are several cytokines and adipokines which can be produced by IPFP in knee OA or patients with ligamentous injury for example IL-6, TNF-α, MCP-1, adiponectin, leptin and visfatin (Klein-Wieringa et al., 2011, Clockaerts et al., 2012, Ushiyama et al., 2003). Expression of these cytokines, and also MMP-13 in IPFP as shown in this study, suggests that these chemical mediators are present in canine IPFP.

There was an upregulation of IL-6 gene expression in IPFP derived from the dogs in our study with CCLD (Figure 4.1B). IL-6 is an important biomarker of inflammation (Heinrich et al., 1990) and increased expression of IL-6 in the IPFP has been associated with knee pain (Witonski et al., 2010). Moreover, previous work in OA patients has detected an increase in IL-6 secretion from IPFP as compared to SC adipose tissue (Klein-Wieringa et al., 2011). Therefore, the increased IL-6 gene expression found in the IPFP of dog stifle joints suggests that this adipose tissue might be inflamed in CCLD. However, down-regulation of adiponectin, leptin and MMP-13 was seen in IPFP of the clinical group as compared to the controls. These findings contradict the results of previous studies, for example a study in human knee joints demonstrated increased leptin expression in IPFP obtained from OA patients as compared to healthy donors (Conde et al., 2014). Moreover, a microarray study

reported upregulation of both adiponectin and leptin transcripts at end-stage of human OA IPFP as compared to the early-stage OA IPFP (Gandhi et al., 2011). However, it is possible that IPFP is not the only source of adipokines in the stifles, since they can be synthesised by other joint tissues such as synovium, osteophytes and bone (Presle et al., 2006). Both pro- and anti-inflammatory mediators are also secreted by IPFP (Ioan-Facsinay and Kloppenburg, 2013); nonetheless, some previous studies have suggested a protective role of IPFP, by reducing catabolic process of cartilage for example in suppressing nitric oxide, glycosaminoglycan release and MMP expression in cartilage (Bastiaansen-Jenniskens et al., 2012). Therefore, the combined effect of these mediators is difficult to predict, and the exact stage of disease may affect the expression of adipokines in IPFP. However, we could not determine exact stage of the CCLD in the CCL samples included in this study.

Additionally, it has been demonstrated that adiponectin stimulates the production of MMP-13 in synovial cells from RA patients to the same degree as IL-1β (Choi et al., 2009), whilst leptin in OA IPFP can enhance expression and production of MMP-1 and MMP-13 in human articular chondrocytes via mitogen-activated protein kinase pathways (Hui et al., 2012). In a similar manner, autocrine effects of adiponectin and leptin in IPFP might be responsible for the concurrence of gene expression of these adipokines and MMP-13 in the current study.

4.4.1.3 Differences in gene expression of pro-inflammatory cytokines and adipokines in disease and non-disease subcutaneous fat

In this work, there were divergent patterns between SC fat and the IPFP when comparing gene expression between cases and controls (except for IL-6 gene expression). Likewise, recent studies have demonstrated that the adipokine gene expression in IPFP correlates more closely with the severity of OA than adipokine gene expression in SC adipose tissue (Gandhi et al., 2015). Dissimilarities in gene expression of different proteins in mesenchymal stem cells derived from IPFP and SC fat have also been described previously (Lopa et al., 2014). The upregulation of both leptin and various inflammatory mediators (IL-6, MCP-1, TNF-α, MMP-13) in SC

tissues of dogs with CCLD that was seen in this study is also noteworthy. Although the reasons for these findings are not known, differences in body condition score might be responsible, not least because it was greater in clinical cases than in control dogs. Leptin is a well-known biomarker of obesity in many species (Hoenig et al., 2007, Ishioka et al., 2007), whilst adipose tissue macrophages can secrete pro-inflammatory cytokines, such as IL-6 and TNF-α, resulting in a low-grade inflammatory state (Ye et al., 2007).

Some limitations of this study should be acknowledged. First, there were differences in the dog breeds represented in the two groups, with most of the control dogs being Staffordshire Bull Terriers, whilst a wider variety of dog breeds, in particularly Retrievers, were recruited for the clinical group. The differences between clinical cases and controls might be due to that the genetic differences amongst breeds (Mankowska et al., 2016). Moreover, most of the clinical dogs were neutered, whereas most dogs in the control group were entire. Not only obesity is more common in neutered dogs (McGreevy et al., 2005), but neuter status can also directly affect plasma adipokine concentrations in dogs (Damoiseaux et al., 2014). Therefore, it is important to be mindful that the results may be affected by these factors.

4.4.2 The role of adipokines in canine CCLD

Previous studies have suggested that adipokines may be predictive biomarkers of joint diseases such as OA and RA (Del Prete et al., 2014, Poonpet and Honsawek, 2014). In contrast, some studies have suggested that adiponectin may have beneficial effect on OA (Chen et al., 2006b, Gandhi et al., 2010), although evidence is conflicting. For example, plasma adiponectin concentrations in RA and OA patients are greater than in the controls (Otero et al., 2006, de Boer et al., 2012), whilst adiponectin concentration in synovial fluid from OA patients correlates with aggrecan degradation markers (Hao et al., 2011). Furthermore, adiponectin can induce nitric oxide synthase type II and pro-inflammatory cytokines in chondrocytes *in vitro* (Lago et al., 2008). In the current study, there was a positive correlation between IPFP adiponectin gene expression and pro-inflammatory cytokine (TNF-α)

concentrations in synovial fluid from the CCLD joints. Although low correlation coefficients and high p-values were noticed, it may be worth considering adiponectin as one of the potential mediators involved in degenerative joint diseases such as CCLD.

In vitro studies have indicated that leptin and visfatin exert pro-inflammatory and pro-catabolic roles in OA (Otero et al., 2003, Gosset et al., 2008). The circulating serum leptin concentration is increased in humans with severe OA (de Boer et al., 2012), whilst leptin concentration in synovial fluid is correlated with joint pain and severity of OA (Ku et al., 2009, Lubbeke et al., 2013). Increased expression of visfatin in the arthritic manus and pes extracts has been demonstrated in mice with collagen-induced arthritis (Evans et al., 2011). Furthermore, visfatin inhibition effectively reduces the severity of disease, circulating TNF-α concentration and pro-inflammatory cytokine secretion in affected joints of this murine model (Busso et al., 2008, Evans et al., 2011). The work presented here has confirmed a positive relationship between synovial fluid leptin concentration and clinical lameness of dogs with CCLD, which were assessed both visually and by force plate measurement. It would have been helpful to have measured synovial fluid visfatin concentration. Unfortunately, it was only possible to collect a small volume of synovial fluid from each dog, and insufficient volumes were available for such measurements.

Another observation of the current study was the variable correlation among some adipose-derived adipokines and cytokines in CCLs. For example, gene expression of IPFP visfatin was positively correlated with gene expression of CCL TNF-α, whereas gene expression of SC visfatin were inversely correlated with gene expression of CCL TNF-α. Previous work has shown that osteophytes release more leptin and visfatin than synovium and IPFP (Presle et al., 2006, Chen et al., 2010). Therefore, adipokines derived from other articular tissues may contribute with mixed effects in the canine knee joint following CCLD. Further studies will be required to clarify this finding. These should include examination of the profile of adipokines in

other joint tissues such as synovium and osteophytes, as well as synovial fluid visfatin concentrations.

4.4.3 Pathway of local and systemic adipose tissues chemical mediators in CCLD

Both local and systemic effects of obesity have been investigated in the development and progression of many diseases such as cancer, osteoarthritis and rheumatoid arthritis (Poonpet and Honsawek, 2014, Iyengar et al., 2015, Senolt, 2017). Central adiposity is suggested to play a major role in the pathogenesis of OA (Wang et al., 2015). For example, plasma adiponectin and leptin concentrations are increased in OA subjects as compared to controls (de Boer et al., 2012), whilst some researchers have emphasised the destructive effects of local inflammatory mediators in OA disease (Clockaerts et al., 2010). In this study, there was an association between the amount of body fat and gene expression for some mediators such as adiponectin, leptin and MMP-13 in SC adipose tissue, but not in IPFP. This finding might suggest that there is a different regulatory pathway for adipokines in local environments compared with systemic adipose tissue.

There were also differences in the patterns of adipokine expression in IPFP and SC tissues from dogs with CCLD and control dogs. These differences might result from varies in the baseline data of dogs recruited in the clinical and the control groups such as age, neuter status and body condition score. Previous studies have described possible effects of various baseline characteristics on adipokines pattern. For instance, plasma adiponectin concentration has been found to be lower in neutered dogs compared to intact dogs and negatively correlated with their age (Damoiseaux et al., 2014).

Previous studies have also shown different patterns of adipokines presented in IPFP and SC adipose tissue in human knee OA (Distel et al., 2009), whilst recent work has suggested a closer association of metabolic activity in IPFP rather than SC in an early stage knee OA (Gandhi et al., 2015). In this study, some IPFP-derived

adipokines were associated with local mediators (for example adiponectin with synovial TNF-α, leptin with CCL MMP-13 and visfatin with CCL TNF-α). In contrast, only SC visfatin was correlated with synovial and CCL TNF-α. Therefore, these findings suggest an independent pathway between local and systemic chemical mediators in pathophysiology of the stifle joint.

Finally, we unexpectedly observed a positive correlation with adiponectin gene expression in SC tissue and body adiposity, which contrasts with previous work suggesting increased fat mass to be inversely associated serum adiponectin concentration (Ricci and Bevilacqua, 2012). However, previous researchers have suggested that visceral adipose tissue, rather than SC adipose tissue, is involved in the secretion of systemic adipokines (Matsuzawa, 2006). Future work, sampling a source of visceral fat would be useful to improve our understanding of this.

4.5 Conclusion

This is the first study to examine the association between adipose tissue adipokines, inflammatory cytokines and ligament tissue degradation biomarkers in CCLD. Our data indicates the presence of inflammation in CCL, IPFP and SC tissues from dogs with CCLD. The contrasting pattern of adipokines expressed in IPFP and SC, as well as the lack of association between body fat mass and the IPFP adipokine profile suggest separate regulatory pathways of fat metabolism in the joint cavity. The various associations of IPFP-expressed adipokines with disease biomarkers in CCLs and joint fluid of dogs with CCLD such as IPFP adiponectin and SC visfatin with TNF-α in synovial fluid or may indicate that local adipokines are more likely to play roles in the disease. Moreover, it is interesting that leptin concentrations in synovial fluid appear to be an indicator for clinical lameness in dogs with CCLD.

Chapter 5 Interaction of adipose tissues and adipokines with cranial cruciate ligaments

5.1 Introduction

The traditional understanding of adipose tissue as a large source of energy and body homeostasis regulator has been challenged since the discovery of leptin (Zhang et al., 1994). Adipose tissue, in particular white adipose tissue, is now identified as an active endocrine organ that synthesises and releases a variety of bioactive peptides. These proteins, termed adipokines, can act through endocrine, paracrine and autocrine mechanisms (Kershaw and Flier, 2004). In recent studies, adipokines have emerged as mediators of adverse health effects within the human musculoskeletal system (Lago et al., 2007a). Adiponectin, leptin and visfatin are well studied adipokines that are involved in inflammatory and immune responses within the body, and these processes become dysregulated in joint disorders such as in osteoarthritis (OA) and rheumatoid arthritis (RA) (Lago et al., 2007b, Toussirot et al., 2007).

Adiponectin is a powerful mediator that enhances the expression of connective tissue biomarkers such as metalloproteinase (MMP)-1 and -13 in synovial cells derived from RA patients (Choi et al., 2009). A recent study also demonstrated that adiponectin can increase nitric oxide, IL-6, MMP-1 and MMP-3 production in chondrocytes and OA cartilage in a mitogen-activated protein kinase-dependent manner (Koskinen et al., 2011).

Various studies have shown that leptin is involved in bone development and cartilage metabolism (Gualillo, 2007, Upadhyay et al., 2015). However, leptin may provide a metabolic link between obesity and OA (Griffin et al., 2009). In severely osteoarthritic patients assessed by radiography, both the concentration of leptin in synovial fluid, and its gene expression in cartilage and joint osteophytes are greater than in tissues from healthy individuals (Dumond et al., 2003, Simopoulou et al.,

2007, Ku et al., 2009). Moreover, leptin can induce the expression of biomarkers for cartilage damage such as MMP-9 and MMP-13 (Hui et al., 2012). Based upon this evidence, leptin is now identified as a key pro-inflammatory factor during joint disease (Toussirot et al., 2007).

A more recent adipokine discovery, the adipokine visfatin has also been examined in musculoskeletal diseases. Clinical studies of human RA patients have demonstrated an association between the visfatin concentrations of both serum and synovial fluid and radiographic evidence of joint damage and inflammation (Brentano et al., 2007, Rho et al., 2009). Furthermore, a role in extracellular matrix-degradation has been suggested. For example, *in vitro* studies have demonstrated a catabolic effect of visfatin, when combined with IL-1, that stimulates the expression of metalloproteinase activity, nitric oxide production, and proteoglycan release in cartilage and meniscus explants (McNulty et al., 2011). Moreover, visfatin also significantly induces the expression of pro-inflammatory cytokines such as interleukin-6 (IL-6) and macrophage chemotactic protein-1 (MCP-1) in chondrocytes and osteoblasts (Laiguillon et al., 2014).

Overall, significant evidence exists to support a key role for adipokines in the breakdown of articular tissues, and this may explain the link with biological effects of obesity in such joint diseases. However, so far no study has focused on the role of adipokines in cruciate ligament pathogenesis. Understanding the effect of adipokines on intra-articular tissue such as the cranial cruciate ligament (CCL) would be advantageous in defining whether they might represent therapeutic targets for ligament breakdown and therefore prevent joint disease. The assessment of biochemical markers of cartilage metabolism such as collagen, extracellular matrix genes, proteases and their inhibitors have been used to detect the CCL injury (Harkey et al., 2015). The expression of matrix metalloproteinase (MMP)-13 gene and aggrecan (AGC) gene are believed to have a role in the pathogenesis of CCL, and would be changed in ruptured CCL when compared to normal CCL (Harkey et al., 2015).

The hypothesis of this chapter was that both systemic and local (intra-articular stifle joint tissues) cross-talk occurs between canine adipokines and articular tissue of dogs and these mechanisms can play a role in the pathophysiology of cranial cruciate ligament.

5.1.1 Aims

- To examine the cross-talk effect of both local and systemic adipose tissues on CCL explants in terms of glycosaminoglycan (sGAG) production.
- To examine the effect of adiponectin, leptin, and visfatin on CCL cells in relation to cartilage degradation biomarkers gene expression, cell viability and migration activities.

5.2 Methods

5.2.1 Alteration of glycosaminoglycan content in CCL tissues

5.2.1.1 Animals

Eight control dogs and six dogs with CCLD (Appendix 3 Table 1-4) were used to obtain CCL, infrapatellar fat pad (IPFP) and subcutaneous (SC) fat explants in this study. Ethical permission for use of tissues and cadavers was approved by the local institutional ethical committees (Chapter 2 Section 2.1).

5.2.1.2 Culture of explants

Intermediately after tissue collection, CCL, IPFP and SC explants were cultured in complete media for 14 days in five different groups: 1) culture of CCL; 2) culture of IPFP; 3) culture of SC; 4) co-culture of CCL and IPFP; 5) co-culture of CCL and SC. Media were collected and changed every 3 days (Chapter 2 Section 2.4.1).

5.2.1.3 Glycosaminoglycan assay

To examine the possible role that adipose tissues and adipokines play in the pathogenesis of CCL tissue, we determined alterations of glycosaminoglycan (sGAG) in ligament explants after 14 days of co-culture with IPFP or with SC. Briefly, sGAG concentration in digested tissues and media of each cultured group were measured using 1,9-dimethylmethylene blue (DMMB)-based spectrophotometry method (Farndale et al., 1982) (Chapter 2 Section 2.4.6).

5.2.2 Investigating gene expression of cartilage degradation markers

5.2.2.1 CCL cells culture

CCL tissues from eight control dogs and six dogs with CCLD (Appendix 3 Table 2) were isolated and cultured in T75 flasks (Chapter 2 Section 2.4.2). CCL cells at first or second passage were then transferred to culture in 24-well plates (Chapter 2 Section 2.4.3).

5.2.2.2 Cell treatment with adipokines

CCL cells at 90% – 100% confluence in 24-well plates were treated in duplicate with recombinant human adiponectin, leptin and visfatin (PeproTech) at different concentrations (0.1 ng/mL, 1 ng/mL and 10 ng/mL), and at three different time points (1 hr, 6 hr and 24 hr) using 1 ng/ml dosage.

5.2.2.3 Measuring gene expression of cartilage degradation markers

Cells were harvested in 1ml of Tri-Reagent following exposure to adipokines at set time points. RNA extraction was performed from CCL cells after which cDNA were synthesised using reverse transcriptase. Then, qPCR was carried out to determine the gene expression for MMP-13 and aggrecan (AGC) in CCL cells and compared to negative controls (Chapter 2 Section 2.5.1.2-2.5.1.4).

5.2.3 Investigating cell viability

CCL cells isolated from six controls and six clinical dogs (Appendix 3 Table 3) were cultured in 96-well flat-bottom white tissue culture plates for 24 hours. The cells were then treated in triplicate with 10 ng/ml or 50 ng/ml of recombinant human adiponectin, leptin and visfatin for 48 hours. In addition, 200 µg/ml of lipopolysaccharide were used as positive controls. Then, cell viability was analysed using a CellTiter-Glo® Luminescent Cell Viability Assay kit according to the manufacturer's protocol (Chapter 2 Section 2.4.4).

5.2.4 Measuring cell migration activity

CCL cells isolated from one control and one clinical dogs (Appendix 3 Table 4) were cultured in collagen gel using a transwell system. The cells were treated with 10 ng/ml of adipokines; adiponectin, leptin and visfatin, in the presence or absence of 20 ng/ml recombinant human platelet-derived growth factor (PDGF-BB; R&D Systems), for 7 days. Cells treated with PDGF alone were used as positive controls. Histological sections of the collagen gels were processed and stained with haematoxylin and eosin. The percentage of migrating cells and maximum migrated distance were measured and compared to both negative and positive controls (Chapter 2 Section 2.4.5).

5.2.5 Statistical analysis

The statistical significance of differences between groups (section 5.2.1 – 5.2.3) was determined using non-parametric Friedman test, followed by the Conover post-hoc test. In addition, 2-way non-parametric ANOVA was used to enable comparison between control and CCLD groups. Statistical software (StatsDirect version 3 for Windows, StatsDirect Ltd., Cheshire, UK) was used for the analysis, and data were considered statistically significant at $P < 0.05$.

5.3 Results

5.3.1 Adipose tissues inhibit proteoglycan synthesis in CCLs

We examined CCL explants from control dogs after 14 days of co-culture with IPFP or SC fat. The sGAG concentration in CCL explants was decreased when co-cultured with IPFP and SC fat, from 8.87 ± 4.20 µg/mg in controls to 5.66 ± 2.60 µg/mg and 6.39 ± 2.82 µg/mg in IPFP (P = 0.039) and SC fat (P = 0.020), respectively (Figure 5.1A). Whilst no significant change in sGAG content was observed between the different experimental groups when explants from dogs with CCLD were examined (Figure 5.1A).

We also determined accumulated sGAG released in the media throughout 14 days of the culture. As with the explant experiments, the concentration of sGAG in the media of the co-culture groups, CCL with IPFP and CCL with SC fat, was decreased from 97.62 ± 45.43 µg/ml to 29.03 ± 28.88 µg/ml and 15.62 ± 13.10 µg/ml respectively (Figure 5.2A). These differences were statistically significant for the media from the co-cultures of CCL and SC fat (P = 0.025, Figure 5.2B). No significant change in sGAG concentration was found in the media from the cultures of the CCLD group (Figure 5.2B). In addition, it was notable that the sGAG concentrations in media form CCLs collected from dogs with CCLD was less than control dogs; 28.43 ± 19.34 µg/ml vs 97.62 ± 45.43 µg/ml (P = 0.006, Figures 5.1B).
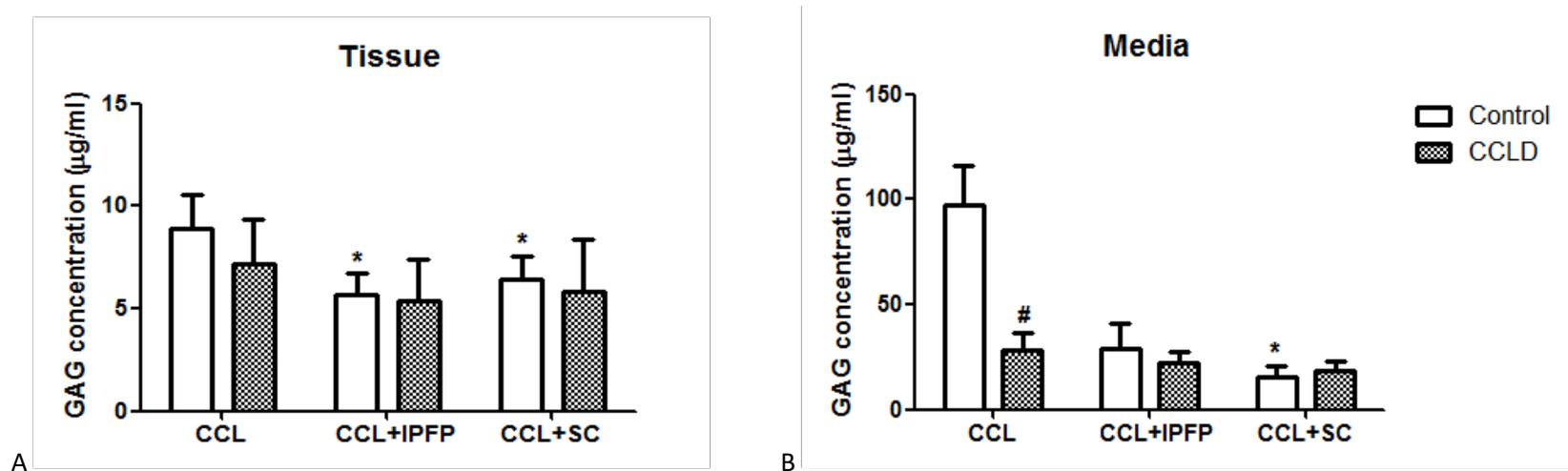
Figure 5.1 sGAG concentrations in different CCL culture groups.

sGAG concentration in explants (A) and media (B) of different CCL culture groups from control dogs and dogs with CCLD. Data are presented as mean and error bars represent the standard error (*P < 0.05 between culture groups, #P < 0.05 between control and CCLD groups).

CCLD: cranial cruciate ligament disease; IPFP: infrapatellar fat pad; SC: subcutaneous fat; CCL+IPFP: CCL that was co-cultured with IPFP; CCL+SC: CCL that was co-cultured with SC fat. Figures show that the sGAG concentration in CCL explants of control dogs was significantly decreased when co-cultured with both IPFP and SC fat (A). sGAG concentration in the media of control dogs was also decreased when co-cultured CCLs with SC fat (B). The sGAG concentrations released in media form CCLs collected from dogs with CCLD is less than control dogs (B). However, there was no significant change in sGAG concentration when explants and media from dogs with CCLD were examined (A-B).

5.3.2 Alteration of cartilage degradation markers in response to adipokines stimulation

Cells isolated from CCL tissue were cultured to form monolayers to determine marker gene responses to adipokine, which were adiponectin, leptin and visfatin, stimulation. A significant increase in MMP-13 gene expression was observed when CCL cells were stimulated with adiponectin at 0.1 ng/ml and 10 ng/ml in the CCLD group (P = 0.011 and P = 0.033 respectively, Figure 5.3B), but not in cells from the control group (Figure 5.2A). No significant differences in response to different concentrations of leptin and visfatin treatment were observed in either control or CCLD group (Figure 5.2B-C). There was no marked change in gene expression of AGC when CCLs were stimulated with different concentrations of adiponectin, leptin or visfatin in either control or CCLD groups (Figure 5.3A-C).
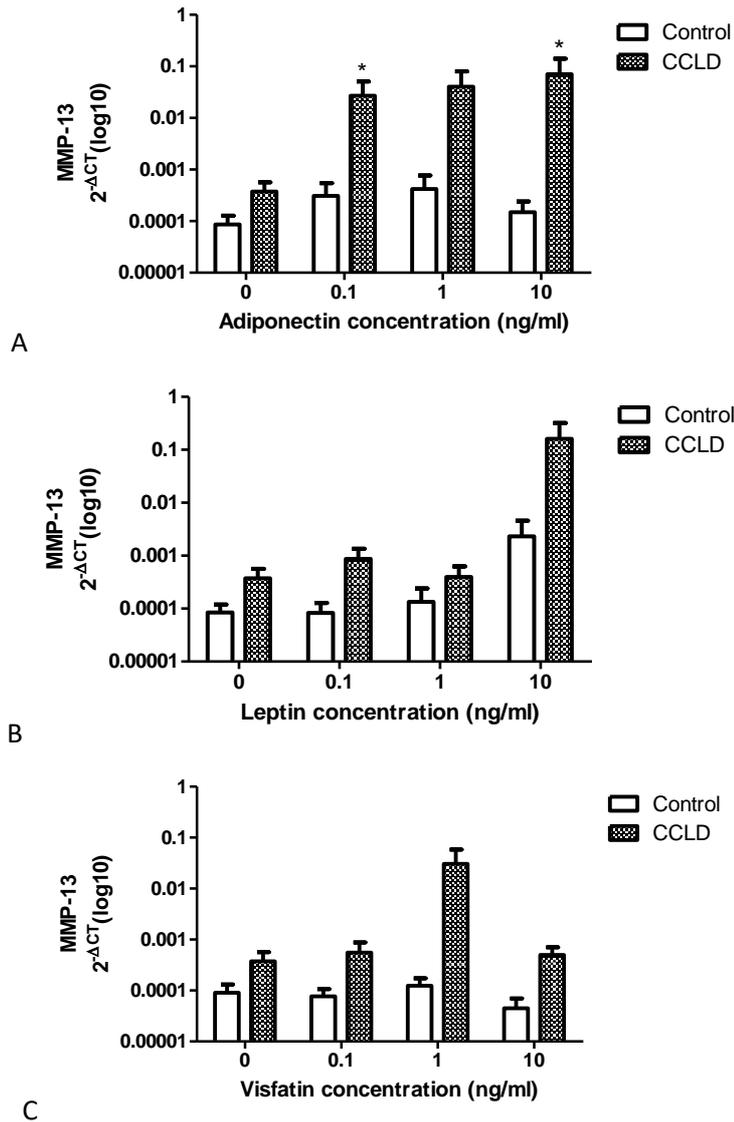
Figure 5.2 MMP-13 gene expression in isolated CCL cells following treatment with different concentrations of adipokines.

Changes in MMP-13 gene expression of CCL cells derived from control and CCLD dogs in response to 0.1 ng/ml, 1 ng/ml and 10 ng/ml of adiponectin (A), leptin (B) and visfatin (C) for 24 hours. Data are presented as mean and error bars represent the standard error (*P < 0.05). CCLD: cranial cruciate ligament disease, MMP-13: matrix metalloproteinase-13. Figures show that MMP-13 gene expression was significantly increased when CCL cells of CCLD group were treated with adiponectin at 0.1 ng/ml and 10 ng/ml (A). There was no significant change in MMP-13 gene expression when CCL cells were treated with different concentrations of leptin and visfatin (B-C).
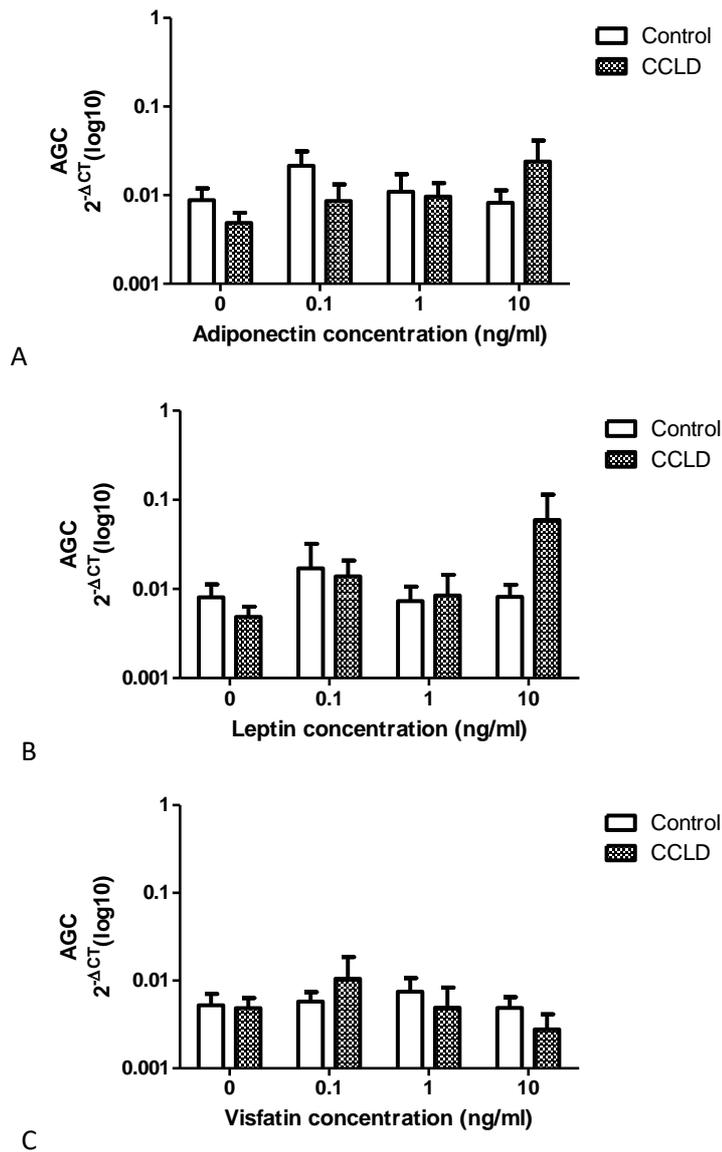
Figure 5. 3 AGC gene expressions in isolated CCL cells following treatment with different concentrations of adipokines.

Changes in AGC gene expression of CCL cells derived from control and CCLD dogs in response to 0.1 ng/ml, 1 ng/ml and 10 ng/ml of adiponectin (A), leptin (B) and visfatin (C) for 24 hours. Data are presented as mean and error bars represent the standard error. CCLD: cranial cruciate ligament disease, AGC: aggrecan. Figures show that there was no significant change in AGC gene expression when CCL cells were treated with different concentrations of any adipokines (A-C).

We examined temporal response to adipokine exposure, and no significant change in MMP-13 gene expression was seen in cells from either the control or CCLD groups (Figure 5.4A-F). However, AGC gene expression in CCL cells from the control group was significantly decreased after 1 hr (P = 0.007), and then increased after 6 hr (P = 0.019) of 1 ng/ml visfatin stimulation (Figure 5.5E). However, there was no alteration in AGC gene expressions in response to visfatin in cells from the CCLD group (Figure 5.5F).
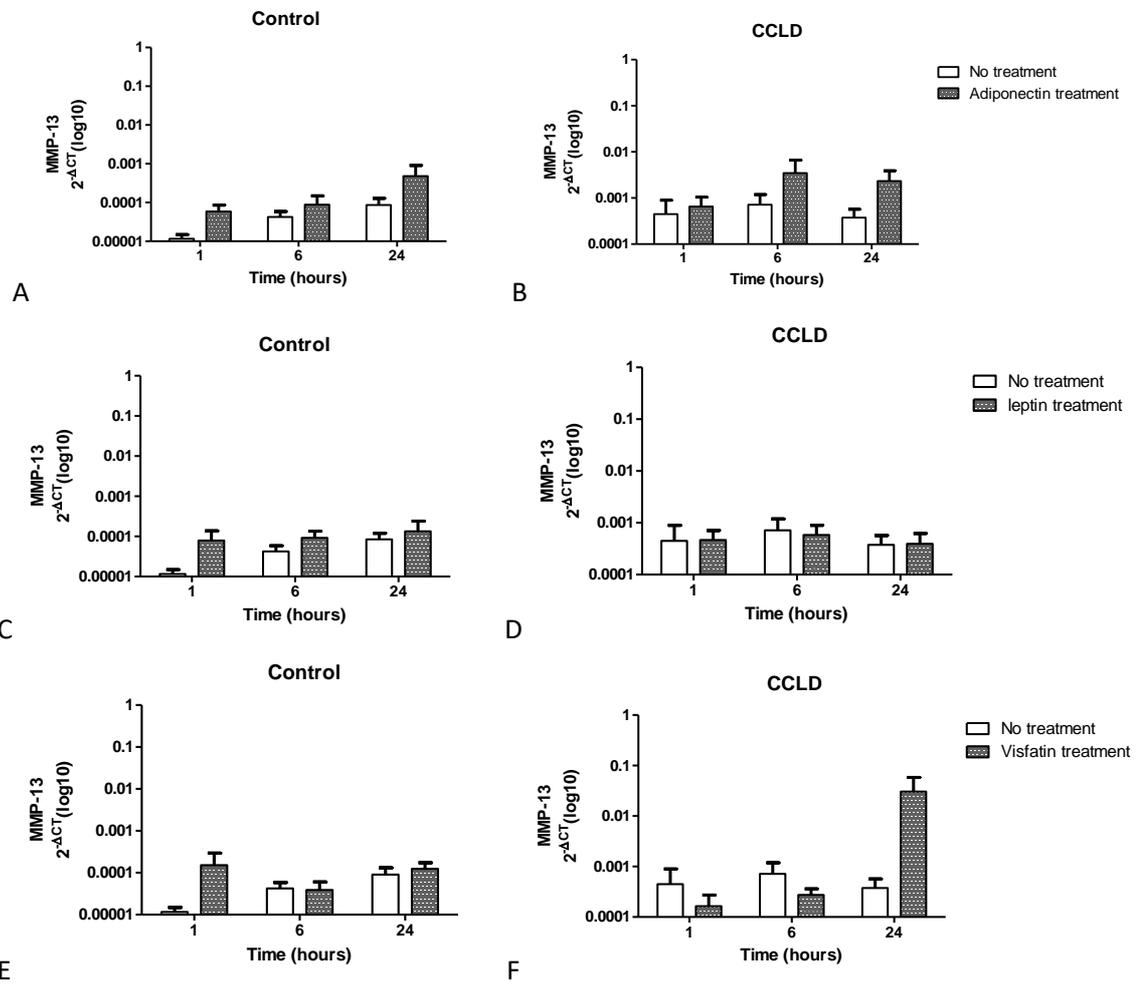
Figure 5. 4 MMP-13 gene expressions in CCL cells following adipokines treatment at different time points.

Changes in MMP-13 gene expressions of CCL cells derived from control (A, C, E) and CCLD (B, D, F) dogs in response to 1 ng/ml of adiponectin (A-B) leptin (C-D) and visfatin (E-F) at different time points. Data are presented as mean and error bars represent the standard error. CCLD: cranial cruciate ligament disease, MMP-13: matrix metalloproteinase-13. Figures show no significant change in MMP-13 gene expression when CCL cells from both control and CCLD group were treated with adipokines over a time course (A-F).
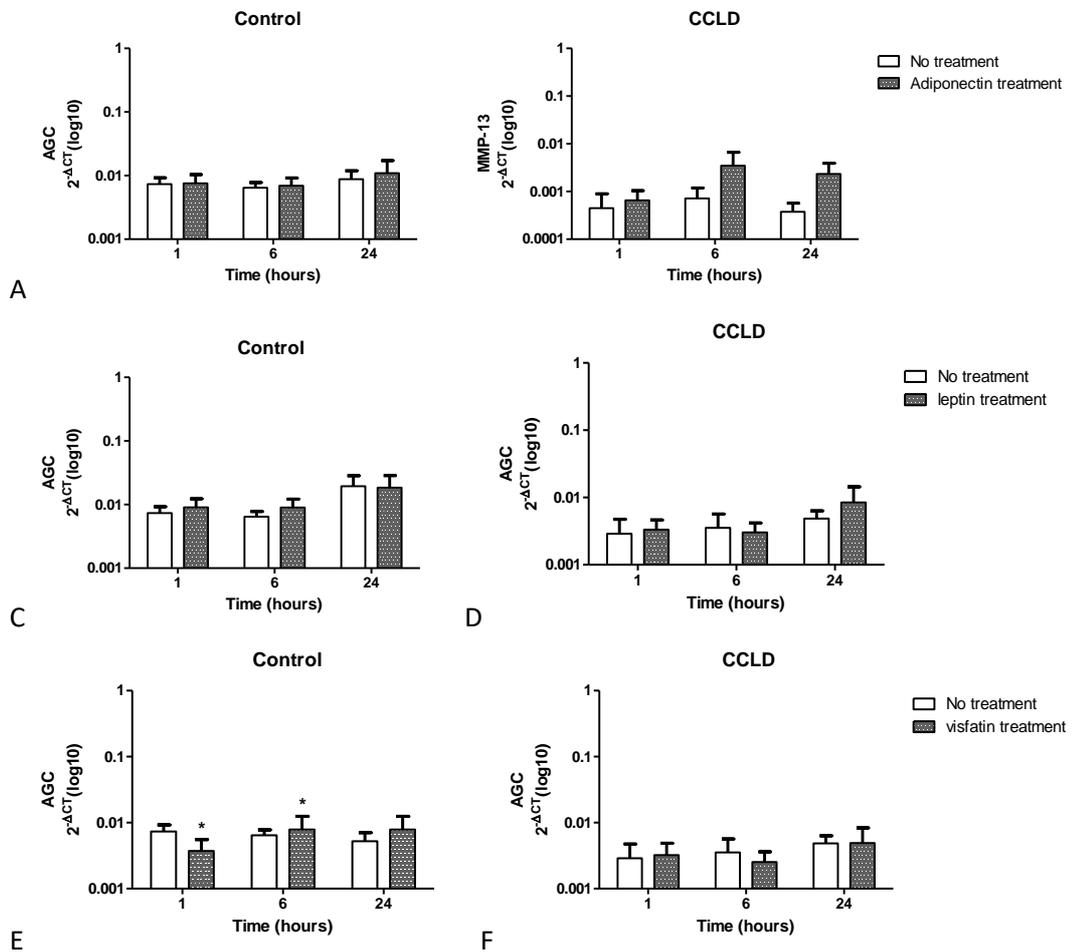
Figure 5.5 AGC gene expressions in CCL cells following adipokines treatment at different time points.

Changes in AGC gene expressions of CCL cells derived from control (A, C, E) and CCLD (B, D, F) dogs in response to 1 ng/ml of adiponectin (A-B) leptin (C-D) and visfatin (E-F) at different time points. Data are presented as mean and error bars represent the standard error. CCLD: cranial cruciate ligament disease. Figures show no significant change in AGC gene expression when CCL cells from CCLD groups were treated with adiponectin, leptin and visfatin at various time points (B, D, F). In the control group, AGC gene expression was significantly decreased when CCL cells were treated with visfatin at 1 hr and increased at 6 hr (E).

5.3.3 Adverse effect of adipokines on CCL cell viability

In the control animals, CCL cell numbers were significantly decreased following treatment with 50 ng/ml adiponectin from 3,000 ± 1,420 cells (8,824 ± 4,177 cells/cm^2) to 2,501 ± 1,413 cells (7,357 ± 4,155 cells/cm^2, P < 0.001, Figure 5.6). Following treatment with 10 and 50 ng/ml of leptin, the cell numbers decreased to 2,687 ± 1,281 cells (7,903 ± 3,769 cells/cm^2, P = 0.012) and 2,504 ± 1,373 cells (7,366 ± 4,039 cells/cm^2, P < 0.001) respectively (Figure 5.6). Following treatment with 10 and 50 ng/ml of visfatin, the cell numbers decreased to 2,675 ± 1,392 cells (7,869 ± 4,093 cells/cm^2, P = 0.001) and 2,292 ± 1,480 cells (6,742 ± 4,353 cells/cm^2, P < 0.001) respectively (Figure 5.6).

Similar results were observed in CCLs cells from dogs with CCLD. Reductions in CCL cell numbers were detected following treatment with 50 ng/ml of adiponectin, 10 ng/ml and 50 ng/ml of leptin, 10 ng/ml and 50 ng/ml of visfatin, from 1,645 ± 1,508 cells (4,839 ± 4,434 cells/cm^2) to 1,446 ± 1,511 cells (4,252 ± 4,444 cells/cm^2, P < 0.001), 1,527 ± 1,500 cells (4,492 ± 4,412 cells/cm^2, P = 0.019), 1,427 ± 1,472 cells (4,197 ± 4,330 cells/cm^2, P < 0.001), 1,453 ± 1,429 cells (4,274 ± 4,202 cells/cm^2, P = 0.002) and 1,374 ± 1,474 cells (4,040 ± 4,335 cells/cm^2, P < 0.001) respectively (Figure 5.6).
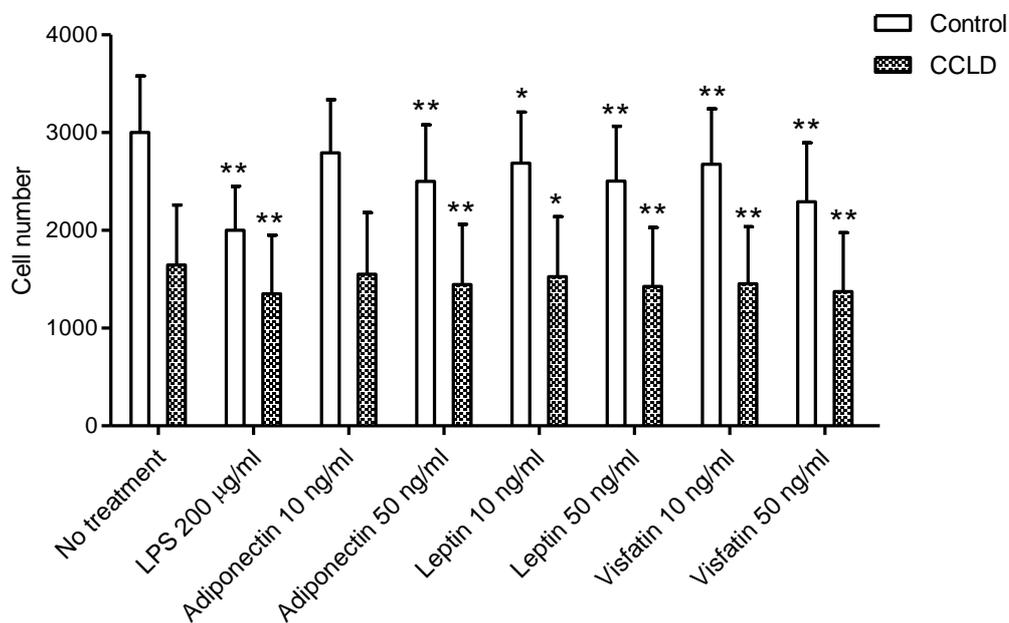
Figure 5.6 CCL cell number following adipokine treatment.

These figures show the number of CCL cells isolated from control and CCLD animals after being exposed to 10 ng/ml or 50 ng/ml of adipokines; adiponectin, leptin and visfatin for 24 hours. LPS at 200 μg/ml was used as a negative control. Data are presented as mean and error bars represent the standard error ($*P < 0.05$, $**P < 0.01$). CCLD: cranial cruciate ligament disease; LPS: lipopolysaccharide. Figures show number of CCL cells were significantly decreased when cells from both control and CCLD group were treated with lipopolysaccharide and adipokines, except for adiponectin treatment at 10 ng/ml.

5.3.4 CCL cell migration activity in response to adipokines

To examine how adipokines affected the migration of CCL cells. Therefore, the migration of CCL cells into collagen gels in the presence or absence of PDGF, a potent stimulator of migration (Figure 5.7A) following exposure to adipokines was examined. The result showed robust invasion of CCL cells into the collagen gel when PDGF was present (Figure 5.7B).
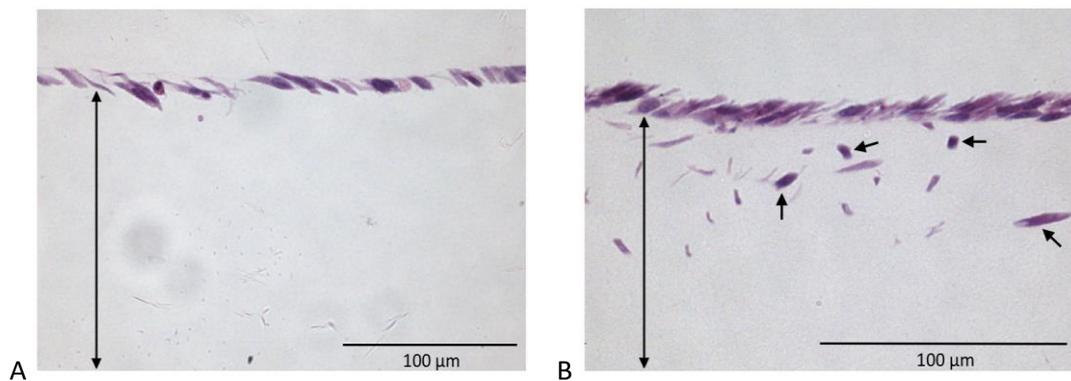
Figure 5.7 Migration activities of CCL fibroblasts.

Cross-sectional images of CCL cells cultured atop three-dimensional collagen gels in either the absence (A) or presence (B) of PDGF (20 ng/ml) for 7 days. The number and the distance of CCL cells invading (one-head arrows) into collagen gel (depth indicated by double-headed arrows) were examined. Figures show no invasion of CCL cells in the absence of PDGF (A), but there was invasion of CCL cells in the presence of PDGF (B).

Given the small number of experiments (one per group), no statistical analysis was performed and therefore conclusions on the results of this experiment were inconclusive. However, data on cell migrating numbers of the control group showed that adiponectin and leptin treatment alone and when combined with PDGF caused a reduction in cell migration from 4.5% to 1.7% and 0% respectively (no PDGF), and from 34.3% to 12.9% and 17.8% respectively (with PDGF), Figure 5.8A. In contrast, visfatin treatment alone increased the number of migrating cells from 4.5% to 13.5% (Figure 5.8A). Visfatin combined with PDGF treatment slightly decreased cells migration number from 34.3% to 29.4% (Figure 5.8A).

In the clinical group, none of the cells in the negative control and leptin treatment groups migrated through the collagen gel, whereas adiponectin and visfatin treated groups induced an increase in 2.6% and 11.1% of cell migration number, respectively (Figure 5.8B). In comparison, combinations of adiponectin and visfatin with PDGF reduced the percentage of CCL cells migrating from 14.8% to 3.2% and 5.9% respectively (Figure 5.8B). Unfortunately, we could not obtain data from leptin combined with PDGF treatment in the clinical cells due to a technical problem.

In terms of the migration distance of CCL cells isolated from control animals, adiponectin treatment alone and with PDGF reduced the distance of cells migrating from 12.9 μm to 7.1 μm, and from 45.8 μm to 34.0 μm respectively (Figure 5.8C). Leptin combined with PDGF treatment also reduced the distance of cell migrating from 45.8 μm to 35.3 μm (Figure 5.8C). In contrast, visfatin treatment alone and with PDGF increased the distance of cell migration from 12.9 μm to 24.1 μm, and from 45.8 μm to 65.5 μm respectively (Figure 5.8C). In addition, the maximum distances of cell migration in the clinical group were 36.8 μm and 38.8 μm in adiponectin and visfatin treatment respectively. With PDGF, adiponectin treatment increased the maximum distance from 28.7 to 48.0 μm. Conversely, visfatin combined with PDGF resulted in the shortest maximum distance at 9.2 μm (Figure 5.8D).
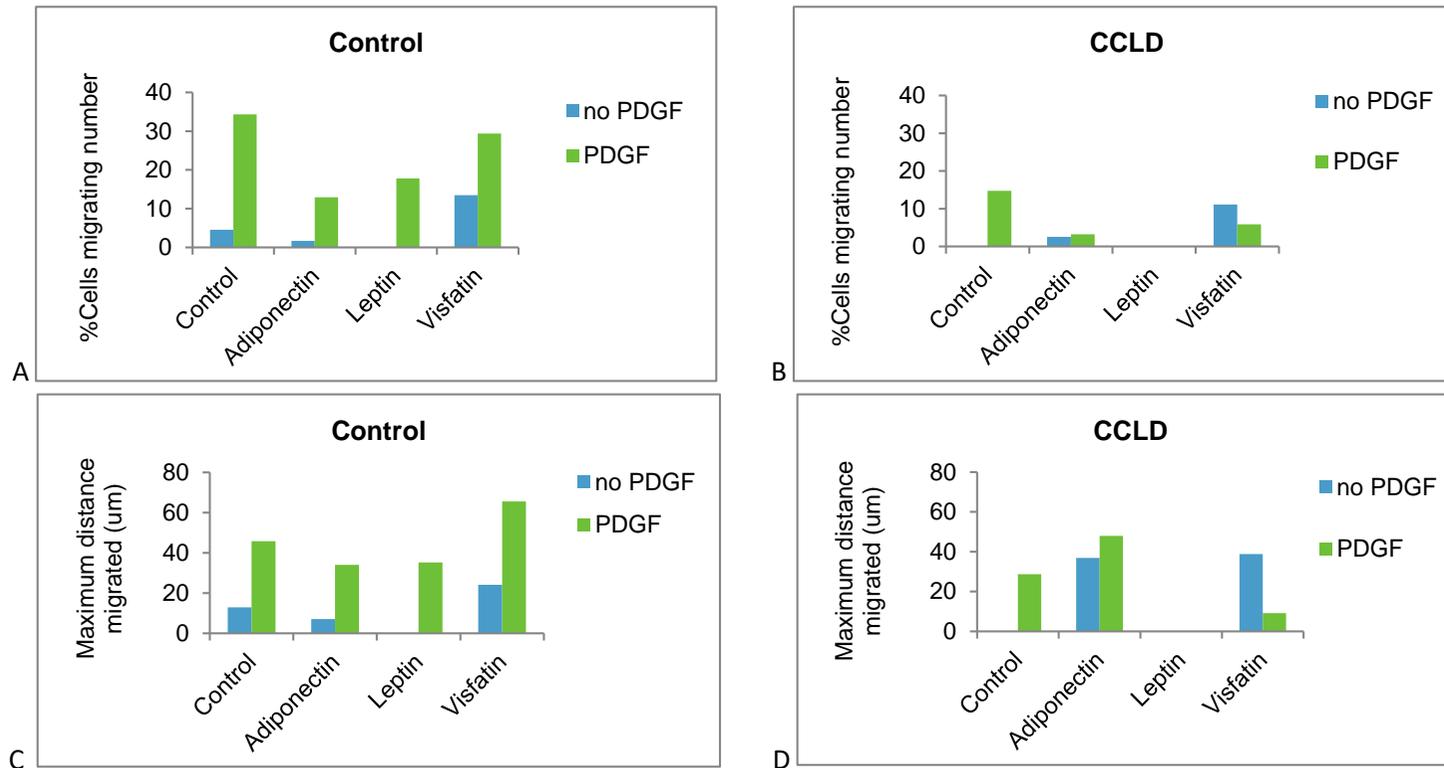
Figure 5.8 The change in CCL cells migration after adipokines exposure.

The migration of CCL cells (%) of control (A) and CCLD group (B); as well as, maximum migrating distance in control (C) and CCLD group (D). CCLD: cranial cruciate ligament disease; PDGF: platelet-derived growth factor. In the control group, decreased cell migrating number and distance migrated was observed in adiponectin and leptin treatment groups, whilst increased cell migrating number and distance migrated in visfatin treatment group were seen visually (A, C). In the CCLD group, decreased cell migrating number but increased distance migrated were seen in adiponectin and visfatin treatment group (B, D). No result was seen for leptin treatment in CCLD group (B, D), and no statistical analysis was performed due to a small sample size (A-D).

5.4 Discussion

5.4.1 Crosstalk effect of adipose tissues on CCL explants

Obesity is one of the primary risk factors for OA in humans (Lago et al., 2007a) and CCLD in dogs (German et al., 2010b). Adipose tissue, the main energy store of the body, is also considered as a source of adipocyte-derived adipokines that can promote joint degeneration (Lago et al., 2007a). This chapter attempted to define the crosstalk between adipose tissues and cruciate ligament tissue using *in vitro* models.

Proteoglycans are present on the cell surface, and within the ECM of most connective tissues, including ligaments (Esko et al., 2009). They have major roles in regulating collagen fibrillogenesis, encouraging tissue hydration and regulating cell to cell signalling, all of which help to maintain the structural integrity of ligaments (Iozzo, 1998). To investigate catabolic effect of adipose tissues on proteoglycans alteration in CCL tissues, concentration of sGAG in the tissues were examined using DMMB. It is noteworthy that the validation of the GAG assay in this study resulted in the negative slope of the DMMB standard curve at 570nm. A previous study has asserted that the ability of the DMMB assay to detect GAG depended on the phenomenon of metachromasia. For example, increasing GAG concentrations resulted in the decline of the 590 nm and 650 nm wavelength and a rise of 525 nm absorbance wavelength (Zheng and Levenston, 2015).

In human studies, sGAG concentration comprised <1% of ACL tissue dry weight for both intact and ACL injury groups (Henninger et al., 2010, Young et al., 2011). Some human and canine studies have shown a difference in the concentrations of sGAG present in normal and ruptured cruciate ligaments, but that was not observed in this study. A study in human ACLs has shown a greater sGAG content in the normal ACL than the injured ACL (Young et al., 2011). In contrast, a study in dogs has demonstrated that sGAG concentrations were increased in injured

CCLs (Comerford et al., 2004). In addition, sGAG content in normal fibrocartilaginous tissue is decreased with increasing age (Wang et al., 2006, Thornton et al., 2015). In the previous canine study, the mean age of the normal group was older than the normal group of this study (7.3 years vs 3.4 years) (Comerford et al., 2004). It may be possible that aging influences sGAG concentration in normal ligaments of dogs leading to the inconsistencies between the studies.

The stage of disease is a factor that can affect the changes in ECM protein concentrations of injured joint tissues (Fu et al., 2007, Catterall et al., 2010). An inverse relationship between proteoglycan concentration in synovial fluid with stage of joint disease such as OA has been reported (Dahlberg et al., 1992). The findings from a human *in vivo* study have shown that acute ACL rupture (3 weeks onset) accelerated the secretion rate of sGAG from femoral weight-bearing cartilage into synovial fluid (Tiderius et al., 2005). It is possible that in our *in vitro* study created acute injury to the normal CCL tissues while the tissues have been cut. Therefore, we observed quite a high concentration of sGAG released in the culture media, compared to the ruptured group. Although we could not trace the exact onset of the CCL disease for every clinical dog in this study, the average disease stage was deemed to be approximately 6 weeks.

In this study, co-culture of CCL explants with adipose tissue reduced the sGAG content of the CCLs, and also reduced sGAG loss from the explants (only in CCL+SC group) through the co-culture period. This phenomenon was only observed in the intact ligaments. Increased matrix turnover in the injured CCLs indicated by increased sGAG synthesis has been demonstrated previously (Comerford et al., 2004). Therefore, it is possible that the injured tissues might be able to compensate for sGAG turnover themselves.

It has been shown that sGAG loss from tissue depends upon the quantity of sGAG remaining in the tissue matrix and the metabolic activity of the cells (Dahlberg et al., 1992, Tiderius et al., 2005). Our study suggests that there might be mediators in adipose tissues that can inhibit proteoglycan production in the ligaments, with

consequent decrease in sGAG release compared to control ligaments that were cultured on their own. Previous work has identified a degradative effect of adipokines on articular tissues. For example, treatment of porcine cartilage and meniscus explants with independent visfatin can enhance sGAG release (McNulty et al., 2011). Moreover, an *in vitro* study demonstrated that intraarticular injection of high-dose leptin into the rat knee depletes proteoglycan in articular cartilage (Bao et al., 2010). In addition, adipokines such as resistin, leptin, or visfatin reduce sGAG content in bovine meniscus explants, while inducing sGAG release into the conditioned media of the cultures (Nishimuta and Levenston, 2015). Therefore, it is possible that, similar to their influence on other articular tissues, adipokines and cytokines produced from adipose tissue may have important roles in catabolic degradation on CCL tissues by reducing sGAG content and inducing sGAG loss in the ligaments. These altered concentrations of proteoglycans are an important consideration as they will alter the biomechanical and functional properties of the ligament (Franchi et al., 2010).

5.4.2 Effect of adipokines on cartilage degradation markers in CCL cells

The evaluation of inflammatory cytokines and biochemical markers of cartilage metabolism have been widely examined in the ACL (Harkey et al., 2015). In this study, we observed the alteration in the gene expression of MMP-13 and aggrecan in CCL cells after treatment with adipokines (adiponectin, leptin and visfatin). Of the MMP family of extracellular proteinases, which are active in degrading extracellular matrix proteins, MMP-13 is a key interstitial collagenase in connective and cartilaginous tissues (Stura et al., 2013). Therefore, it is commonly monitored in various pathological conditions including ligament tears and tendinopathy (Arnoczky et al., 2007, Xie et al., 2013). Our results showed that adiponectin (0.1 and 10 ng/ml) stimulated MMP-13 expression in CCL cells derived from the clinical group. Previous studies support our finding that adiponectin might contribute to synovitis and joint destruction by strongly stimulating MMP-1 and MMP-13 expression in synoviocytes from RA patients (Choi et al., 2009).

Other studies have found that leptin used at higher dosages than in our study (20 - 500 ng/ml) can induce the expression of MMP-13 in articular chondrocytes from OA patients (Pallu et al., 2010, Hui et al., 2012). In addition, it has been shown that median leptin concentration in synovial fluid of patients with knee OA is 42.08 ng/ml (Calvet et al., 2016). Taken together, concentration of leptin may have needed to be considerably higher than the concentration used in our study to see an effect on MMP-13 expression. This may explain why no change was observed by using $0.1 - 10$ ng/ml dosages.

Aggrecan is an important proteoglycan, found in articular cartilage and cruciate ligaments, which provides tissues with load-bearing properties (Kiani et al., 2002, Valiyaveettil et al., 2005). Preliminary studies suggest that aggrecan can be used as a biomarker of knee injuries and OA (Ahlen et al., 2015). Aggrecan synthesis markers are increased in the synovial fluid, serum and urine of patients with acute ACL injury (Svoboda et al., 2013, Struglics et al., 2015). Moreover, in painful tendinopathy, it has been reported that aggrecan mRNA expression was increased compared with normal tendons (Corps et al., 2006). Our results indicated that aggrecan gene expression in CCL cells from control animals was reduced after a short period (at 1 hr) of treatment with 1 ng/ml of visfatin. This agrees with previous studies, which demonstrated that visfatin down-regulated aggrecan expression, and decreased high molecular weight proteoglycans synthesis in mouse articular chondrocytes (Gosset et al., 2008). Another recent study has also shown that visfatin stimulated the expression of MMPs (MMP-3, MMP-12 and MMP-13) and down-regulated aggrecan (Yang et al., 2015). Therefore, our study supports the evidence that visfatin exerts its catabolic function by down-regulating aggrecan in CCL cells, which may contribute to ligament degradation. Future work to confirm the effect of adipokines could involve increasing the dosage of adipokine treatment, and observing other matrix degradation-related biomarkers such as IL-6, MCP-1, MMP-2 and MMP-9 (Stannus et al., 2010, Boland et al., 2014, Xu et al., 2015).

5.4.3 Effect of adipokines on CCL cell viability

It has been suggested that adipokines are important for cell survival in many tissues (Iwamoto et al., 2004, Nepal and Park, 2015). This study indicates a dose-dependent, detrimental effect of adiponectin, leptin and visfatin on CCL cell viability in both normal and injured groups. Consistent with this, previous studies have demonstrated that adiponectin induces both apoptosis and proliferation of human liver cells (Nepal and Park, 2015). However, this adipokine may have a beneficial effect on cell proliferation in other cell types, such as human osteoblasts (Luo et al., 2005), the mouse ATDC5 cell line (Challa et al., 2010) and human periodontal ligament cells (Nokhbehsaim et al., 2014a). Additionally, previous research has shown that leptin decreases normal and OA chondrocyte proliferation (Simopoulou et al., 2007, Wang et al., 2016). In contrast, some studies have implied cell proliferation promoting effect of leptin in other tissues, such as mouse pancreatic beta cells (Tanabe et al., 1997), human iliac crest osteoblasts (Gordeladze et al., 2002), human dental stem cells (Um et al., 2011) and human liver cells (Nepal and Park, 2015).

Like other adipokines, visfatin can have either a positive or negative role on cell viability, depending upon the circumstances. For example, visfatin enhances proliferation of cultured human osteoblasts (Xie et al., 2007), and stimulates breast cancer cell proliferation (Park et al., 2014). However, it can also increase rates of apoptosis in rat intestinal mucosa (Zhou et al., 2017). Overall, although adipokines have a variety of effects on cell survival in different cell types, this study supports an adverse effect of adiponectin, leptin and visfatin on canine CCL cell viability. Further investigation into the pathway of adipokines action on cell death may identify therapeutic targets in the treatment of CCLD.

5.4.4 Effect of adipokines on CCL cell migration

The migratory property of cells can be an indicator for healing ability following tissue damage (Valluru et al., 2011). A recent study has observed a prominent effect

of adipocyte-conditioned media in induction of human vascular smooth muscle cell migration (Lamers et al., 2011). There is evidence that adiponectin stimulates endothelial cell migration and differentiation, which results in stimulated blood vessel growth *in vivo* in mouse Matrigel® plug implantation and in rabbit corneal models of angiogenesis (Ouchi et al., 2004). In contrast, leptin decreases the number of migrating chondrogenic progenitor cells (Zhao et al., 2016), and negatively interferes with the regenerative capacity of periodontal healing in an *in vitro* wound healing model (Nokhbehsaim et al., 2014b). Furthermore, visfatin has a promoting effect in cell migration capability of human umbilical vein endothelial cells (Xiao et al., 2009).

The data presented in this migration study were pilot results performed during a three-week period, while the researcher (WS) was attending a student exchange programme in Dr.Christopher Mendias's laboratory at the University of Michigan, USA. Due to time constraints, only a limited number of experiments could be run. The subjective differences in migration patterns when stimulated with different adipokines might suggest that they influence CCL cell migration, and have a potential role in the healing ability following the CCL damage.

Some limitation of these studies was that using adipokine's vehicle for the controls, instead of no treatment, would give more precise results. Also, more samples and further work are required to better clarify if and how adipokines from adipose tissue impact CCL tissue.

5.5 Conclusion

This is the first study to demonstrate an influence of adipose tissues and adipokines on cruciate ligament biology. The explant co-culture system demonstrated a reduction in the sGAG content in intact ligaments cultured with both local (IPFP) and systemic (SC fat) adipose tissues. Adiponectin and visfatin played roles in regulating MMP-13 and aggrecan gene in CCL cells respectively. In addition, adiponectin, leptin and visfatin had adverse effects on ligament cell viability. Overall, these results suggest an overall catabolic impact of adipokines on CCL tissues and cells, and provide an important first step in understanding the potential role of adipokines in regulating cruciate ligament metabolism. However, these adipokines may have different effects in the context of normal and pathological ligament tissues, and the findings must therefore be evaluated further. Understanding these mechanisms may point us toward therapeutic targets for disrupting the pathological mechanisms that lead to cruciate ligament disease.

Chapter 6 Final discussion

6.1 General discussion

Cranial cruciate ligament disease (CCLD) is one of the most common musculoskeletal diseases in dogs, and a major cause of stifle osteoarthritis (OA). The disease has important consequences both in terms of morbidity and cost associated with its management. Despite this, there are many gaps in understanding of its aetiology and pathogenesis which in turn means that preventive strategies and therapy are far from perfect. Obesity is known to be an important risk factor of CCLD (Adams et al., 2011). White adipose tissue has recently been recognised to be an active endocrine organ that synthesises and releases various adipokines and cytokines. In humans, these chemical mediators are thought to promote musculoskeletal impairment such as OA and rheumatoid arthritis (RA) (Lago et al., 2007a). This is the first study to investigate the association between adipose tissue and canine CCLD, through both mechanical and humoral factor mechanisms. Specifically, dogs with CCLD were studied to examine the link between adiposity and clinical presentation. We further investigated roles of local and systemic adipokines in the CCL and other tissues of the stifle joint. In addition, we performed *in vitro* experiments to examine the direct effects of adipose tissue and adipokines on CCL tissues and cells.

6.1.1 Impact of obesity on clinical presentation of dogs with CCLD

One of the main questions examined in this study was to determine the influence of obesity-related factors on the clinical presentation of dogs with CCLD. One finding was that excess bodyweight was associated with the severity of lameness, perhaps suggesting a role for increased mechanical loading in pathogenesis of CCLD. This would have implications both for dogs of large breeds not least those predisposed to CCLD (Witsberger et al., 2008). Whilst this association might also have been the result of excessive body fat mass, the lack of association

with body fat mass (measured by DEXA) makes this unlikely. That said, the population studied was small and numbers may have been insufficient to separate a possible effect of body fat mass from the breed effect. This association between bodyweight and severity of lameness in dogs with CCLD agrees with findings from studies of the human knee, whereby the repetitive effect of mechanical loading may lead to weakness and degeneration of the cruciate ligament (Johnson and Johnson, 1993, Moore and Read, 1996c). Moreover, joint pain is associated with synovial fluid leptin concentrations in human with end-stage knee OA (Lubbeke et al., 2013) and it was observed in our study that the concentration of synovial fluid leptin was also positively associated with the degree of lameness in dogs with CCLD. Taken together, these results suggest a contribution from both mechanical and humoral factors in the clinical presentation in dogs with CCLD. Further studies should now be considered to investigate these observations further, and also to determine whether mechanical and humoral effects are inter-related.

6.1.2 Effect of adipose tissue and adipokines on the biology of the cranial cruciate ligament

The novel findings were observed in our *in vitro* studies, which suggest possible biological interactions between adipose tissue and the canine CCL in both health and disease. In this respect, our co-culture experiments identified that adipose tissue may have a catabolic effect on glycosaminoglycan content in CCL explants. In addition, the direct effects of recombinant adipokines (adiponectin, visfatin) on CCL cell gene expression for MMP-13 and aggrecan was examined. These results are agree with those of previous studies which have also suggested a degradative effect of adipokines on glycosaminoglycan content of articular cartilage in rats (Bao et al., 2010), pigs (McNulty et al., 2011) and cattle (Nishimuta and Levenston, 2015).

Relationships amongst adipokines and disease biomarkers were also identified within the canine stifle joint of dogs with CCLD. For example, an association was identified between the gene expression of both infrapatellar fat pad (IPFP) adiponectin and subcutaneous (SC) fat visfatin and concentration of TNF-α in synovial

fluid. There were also associations between IPFP leptin gene expression and CCL MMP-13 gene expression and between both IPFP and SC visfatin gene expression and CCL TNF-α gene expression, which suggest degradation and inflammation process. Other studies have shown that adiponectin can induce both gene and protein expression of chemokines and cytokines (e.g. IL-6 and MMP-3) in synovial fibroblasts of rheumatoid arthritis patients (Frommer et al., 2010). Plasma adiponectin and leptin concentrations were also increased in the knee joint of OA subjects as compared to healthy subjects (de Boer et al., 2012). Visfatin is an important regulator of inflammation, cartilage catabolism and bone erosion (Busso et al., 2008, Evans et al., 2011), whilst recent studies have suggested that adipokines may regulate cell proliferation and cell death in various cell types (Iwamoto et al., 2004, Nepal and Park, 2015). This study has also shown a potential for adipokines (adiponectin, leptin, visfatin) to have adverse effects on ligament cell viability in dose-dependent manner suggesting that adipokines might be an important factor for CCL cell survival. However, further studies would be required to confirm these findings, not least to determine the mechanism for any such effects.

6.1.3 Tentative pathway of the role of adipokines in the canine stifle joint

This study investigated whether the basal production of adipokines and cytokines by local and systemic adipose tissue correlated with body fat mass. Correlations were identified between body fat mass and adipokine profiles in systemic (SC fat), but not in local (IPFP) adipose tissue. Moreover, discrepant results were identified in the gene expression of adipokines and others disease biomarkers between local and systemic adipose tissues. For example, compared with control dogs, leptin and MMP-13 were down-regulated in IPFP but up-regulated in SC of dogs with CCLD. In addition, although it is suggested that central adiposity plays a role in the pathogenesis of OA, some studies suggest that intra-articular mediators have a greater influence (Clockaerts et al., 2012). Differences between the genes expression present in IPFP and SC adipose tissues, as well as a closer association between

metabolic activity in the IPFP and gene expression of disease biomarkers than in the SC fat of OA patients have also been shown previously (Lopa et al., 2014, Gandhi et al., 2015). This study observed the relationship between various IPFP-derived adipokine gene expression (e.g. adiponectin, leptin, visfatin) and CCLD biomarkers in both synovial fluid and CCLs. In contrast, only SC fat visfatin gene expression was correlated with synovial and CCL TNF-α. Taken together, these results suggest independent pathways of regulation for adipokines expressed by local and systemic adipose tissue depots, with local adipokines having more of an influence in the pathogenesis of CCLD.

6.1.4 Limitations of the study

Although many of the aims of the current study were fulfilled, there were some limitations which should be mentioned. First, the baseline data of dogs recruited in the clinical and the control groups were different, for example: breed, neuter status, age, bodyweight and body condition score. Previous studies have described possible effects of various baseline characteristics on adipokines and disease biomarker activity. For instance, higher pro-MMP-2 concentrations have been demonstrated in Labrador Retriever CCLs as compared to Greyhound CCLs (Comerford et al., 2005). Increased expression of MMP-3 has also been found in CCLs from older Hounds, when compared with young Beagles (Muir et al., 2005). A sex-specific difference has also been observed with some adipokines; for example, higher leptin concentration in synovial fluid from women with knee OA as compare to men (Presle et al., 2006). Moreover, plasma adiponectin concentration has been found to be lower in neutered dogs compared to intact dogs and negatively correlated with their age (Damoiseaux et al., 2014). Therefore, overall differences in animal signalment might be an alternative explanation for differences in findings from cases and controls. In further studies, more dogs with tighter signalment could be recruited to enable the effects of confounding to be explored statistically.

A second limitation is that the fact that the stage of disease, such as partial and complete rupture, might have varied amongst different cases and this might

again have influenced gene expression pattern in our samples. In a similar manner, alterations in gene and ECM protein concentrations in adipose tissue and injured tissue at different stages of OA have been reported in humans (Catterall et al., 2010, Gandhi et al., 2015).

We also measured adipokine profiles in SC adipose tissue and this might not have been representative of all systemic adipose tissue depots, not least since given the suggestion that visceral adipose tissue might have a greater influence on systemic adipokine profiles (Matsuzawa, 2006). SC fat was chosen because it was easily accessible and we used a standardised site that had been used before (Ryan et al., 2010). Therefore, in future studies, concurrent assessment of adipokine profiles in blood circulation, in visceral fat, as well as in other joint tissues should be considered.

The use of cadavers to source control tissues meant that it was not possible to measure body composition by DEXA and, instead, only BCS was assessed. In this respect, different results can be obtained when measuring body composition by DEXA in cadavers and live (Lefebvre et al., 1999, Pearce et al., 2009). In addition, using cadavers as a control group for live animals might create some differences in terms of gene expression in body tissue, since it has been shown that there are some changes in expression of oxidative stress regulating genes between human cadaveric and live donor kidney tissues (Kainz et al., 2004).

There was relatively poor inter-observer agreement for radiographic score, which contrasts with previous work using the same scoring system (Innes et al., 2004). This is likely to be due to differences in the experience of the two observers, as previously suggested (Verhoeven et al., 2007). Choosing different subjective scoring systems; for example, using either 30-factor (Hurley et al., 2007) or 21-factor (Au et al., 2010) scoring systems, which include measuring different points of joint tissues such as soft tissue thickening, intraarticular mineralisation and stifle joint effusion. Those more strict classification instructions may help to improve both reader consistency, and also inter-observer agreement (Linney et al., 2011).

Moreover, it has also been suggested that observers are required to implement the scoring system the same way each time (Lee et al., 2015).

It need to be mentioned that a problem of statistical analysis in this study might occur with multiple comparison when correlations among more than two groups were assessed by Kendall' tau test (Table 3.4, 4.3, 4.4). The Bonferroni method should be employed as a post-hoc procedure of correcting p values to avoid a type I error in this circumstance (Armstrong, 2014).

Finally, there were limitations in the volume of synovial fluid samples, so we could not test visfatin concentration. In addition, low numbers in the cell migration experiment, which made it impossible to perform statistical analysis, also needs to be mentioned. Therefore, more samples should be collected in future studies.

6.2 Conclusion

This thesis provides initial steps in exploring the role of adipose tissue in regulating canine cruciate ligament metabolism both in health and disease. The study has identified the potential for both mechanical and humoral factors of obesity in CCLD severity, together with the disease activity in the stifle joints, and suggests a possible role of adipose tissue and adipokines in the aetiopathogenesis of CCLD. Additional findings include the possibility of an independent regulatory pathway of fat metabolism in the joint cavity, given closer associations with the disease biomarkers in CCL when compared to systemic adipose tissue. *In vitro* studies have also indicated cross-talk and catabolic effects of adipose tissues and adipokines on the CCL tissue explants and cells. Understanding how CCLD is affected by obesity, adipose tissue and adipokine will be beneficial for the disease prevention, management and therapeutic interventions.

6.3 Future work

There are several opportunities of further study that could be considered to expand the work undertaken in this thesis. For example, the positive correlation between lameness and synovial fluid leptin concentration observed in this work suggests that leptin might be a biomarker for lameness in dogs or involved in the disease severity. Therefore, it might be worth to test the serum leptin to confirm this effect.

There might also be opportunities for therapeutic intervention, perhaps to influence the effect of adipokine on CCL tissue biology, for example by interfering with the effect of adipokine action on tissue degradation biomarkers in CCL tissue and cell death.

Finally, the results of this study could be a comparative model for exploring influence of adipose tissue on pathogenesis of cruciate ligament disease in humans.

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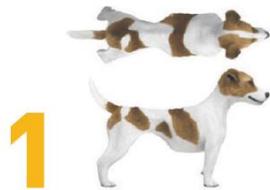
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Appendix 1: Body condition score system for dogs

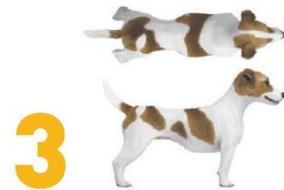# Small dogs body condition score (copyright Royal Canin)

TOO THIN

1
- Ribs, lumbar vertebrae, pelvic bones and all bony prominences evident from a distance
- No discernible body fat
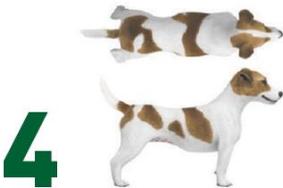- Obvious loss of muscle mass

2
- Ribs, lumbar vertebrae, pelvic bones easily visible
- No palpable fat
- Some bony prominences visible from a distance
- Minimal loss of muscle mass

3
- Ribs easily palpable and may be visible with no palpable fat
- Tops of lumbar vertebrae visible, pelvic bones becoming prominent
- Obvious waist and abdominal tuck

IDEAL

4
- Ribs easily palpable with minimal fat covering
- Waist easily noted when viewed from above
- Abdominal tuck evident

5
- Ribs palpable without excess fat covering
- Waist observed behind ribs when viewed from above
- Abdomen tucked up when viewed from side

OVERWEIGHT

6
- Ribs palpable with slight excess of fat covering
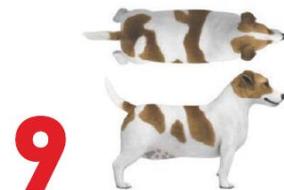- Waist is discernible when viewed from above but is not prominent
- Abdominal tuck apparent

OBESE

7
- Ribs palpable with difficulty, heavy fat cover
- Noticeable fat deposits over lumbar area and base of tail
- Waist absent or barely visible
- Abdominal tuck may be absent

8
- Ribs not palpable under very heavy fat cover or palpable only with significant pressure
- Heavy fat deposits over lumbar area and base of tail
- Waist absent
- No abdominal tuck
- Obvious abdominal distension may be present

9
- Massive fat deposits over thorax, spine and base of tail
- Waist and abdominal tuck absent
- Fat deposits on neck and limbs
- Obvious abdominal distension

171

Medium dogs body condition score (copyright Royal Canin)

TOO THIN

1
- Ribs, lumbar vertebrae, pelvic bones and all bony prominences evident from a distance
- No discernible body fat
- Obvious loss of muscle mass

2
- Ribs, lumbar vertebrae, pelvic bones easily visible
- No palpable fat
- Some bony prominences visible from a distance
- Minimal loss of muscle mass

3
- Ribs easily palpable and may be visible with no palpable fat
- Tops of lumbar vertebrae visible, pelvic bones becoming prominent
- Obvious waist and abdominal tuck

IDEAL

4
- Ribs easily palpable with minimal fat covering
- Waist easily noted when viewed from above
- Abdominal tuck evident

5
- Ribs palpable without excess fat covering
- Waist observed behind ribs when viewed from above
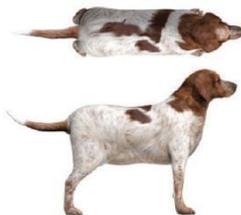- Abdomen tucked up when viewed from side

OVERWEIGHT

6
- Ribs palpable with slight excess of fat covering
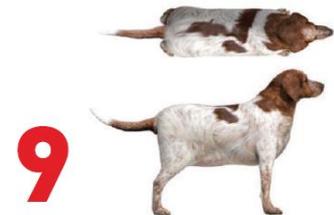- Waist is discernible when viewed from above but is not prominent
- Abdominal tuck apparent

OBESE

7
- Ribs palpable with difficulty, heavy fat cover
- Noticeable fat deposits over lumbar area and base of tail
- Waist absent or barely visible
- Abdominal tuck may be absent

8
- Ribs not palpable under very heavy fat cover or palpable only with significant pressure
- Heavy fat deposits over lumbar area and base of tail
- Waist absent
- No abdominal tuck
- Obvious abdominal distension may be present

9
- Massive fat deposits over thorax, spine and base of tail
- Waist and abdominal tuck absent
- Fat deposits on neck and limbs
- Obvious abdominal distension

172

Large dogs body condition score (copyright Royal Canin)

TOO THIN

1
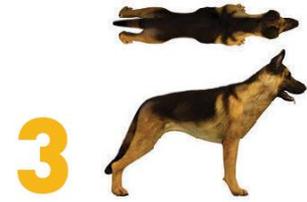- Ribs, lumbar vertebrae, pelvic bones and all bony prominences evident from a distance
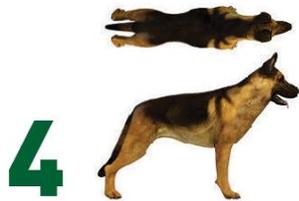- No discernible body fat
- Obvious loss of muscle mass

2
- Ribs, lumbar vertebrae, pelvic bones easily visible
- No palpable fat
- Some bony prominences visible from a distance
- Minimal loss of muscle mass

3
- Ribs easily palpable and may be visible with no palpable fat
- Tops of lumbar vertebrae visible, pelvic bones becoming prominent
- Obvious waist and abdominal tuck

IDEAL

4
- Ribs easily palpable with minimal fat covering
- Waist easily noted when viewed from above
- Abdominal tuck evident

5
- Ribs palpable without excess fat covering
- Waist observed behind ribs when viewed from above
- Abdomen tucked up when viewed from side

OVERWEIGHT

6
- Ribs palpable with slight excess of fat covering
- Waist is discernible when viewed from above but is not prominent
- Abdominal tuck apparent

OBESE

7
- Ribs palpable with difficulty, heavy fat cover
- Noticeable fat deposits over lumbar area and base of tail
- Waist absent or barely visible
- Abdominal tuck may be absent

8
- Ribs not palpable under very heavy fat cover or palpable only with significant pressure
- Heavy fat deposits over lumbar area and base of tail
- Waist absent
- No abdominal tuck
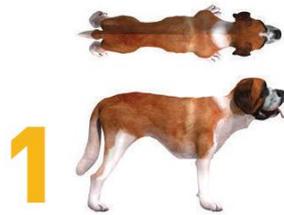- Obvious abdominal distension may be present

9
- Massive fat deposits over thorax, spine and base of tail
- Waist and abdominal tuck absent
- Fat deposits on neck and limbs
- Obvious abdominal distension
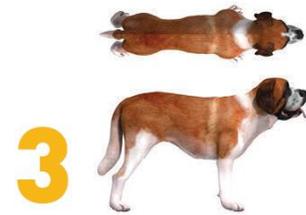
Giant dogs body condition score (copyright Royal Canin)

TOO THIN

1
- Ribs, lumbar vertebrae, pelvic bones and all bony prominences evident from a distance
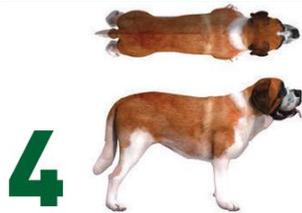- No discernible body fat
- Obvious loss of muscle mass

2
- Ribs, lumbar vertebrae, pelvic bones easily visible
- No palpable fat
- Some bony prominences visible from a distance
- Minimal loss of muscle mass

3
- Ribs easily palpable and may be visible with no palpable fat
- Tops of lumbar vertebrae visible, pelvic bones becoming prominent
- Obvious waist and abdominal tuck

IDEAL

4
- Ribs easily palpable with minimal fat covering
- Waist easily noted when viewed from above
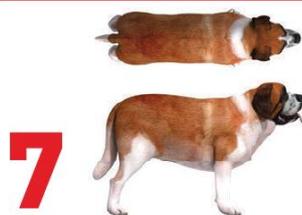- Abdominal tuck evident

5
- Ribs palpable without excess fat covering
- Waist observed behind ribs when viewed from above
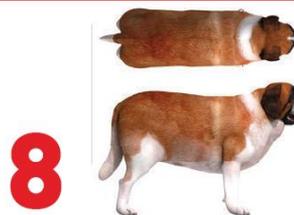- Abdomen tucked up when viewed from side

OVERWEIGHT

6
- Ribs palpable with slight excess of fat covering
- Waist is discernible when viewed from above but is not prominent
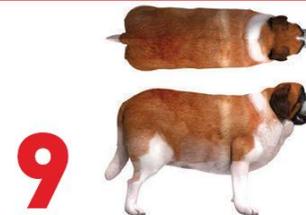- Abdominal tuck apparent

OBESE

7
- Ribs palpable with difficulty, heavy fat cover
- Noticeable fat deposits over lumbar area and base of tail
- Waist absent or barely visible
- Abdominal tuck may be absent

8
- Ribs not palpable under very heavy fat cover or palpable only with significant pressure
- Heavy fat deposits over lumbar area and base of tail
- Waist absent
- No abdominal tuck
- Obvious abdominal distension may be present

9
- Massive fat deposits over thorax, spine and base of tail
- Waist and abdominal tuck absent
- Fat deposits on neck and limbs
- Obvious abdominal distension

Appendix 2: Radiographic assessment of osteophytosis

Score 0

Score 1

Score 2

Score 3

Appendix 3: Animal profile used in Chapter 5

Table 1 Animal profile used in Chapter 5 Section 5.2.1

Category	Control (n=6)		Clinical (n=6)	
	Median	(Range)	Median	(Range)
Breed (n)	Siberian Husky (1)		American Bulldog (1)	
	Staffordshire Bull Terrier (5)		Border Terrier (1)	
			Cross Breed (1)	
			Labrador Retriever (2)	
			West Highland White Terrier (1)	
Gender (n)	Entire female (2)		Entire female (2)	
	Entire male (4)		Neutered female (2)	
			Neutered male (2)	
Age (mt)	42.0	(24.0 – 60.0)	69.0	(36.0 – 97.0)
BCS (0/9 - 9/9)	5	(2 – 5)	6	(6 - 7)
Bodyweight (kg)	22.7	(15.0 – 37.0)	30.0	(9.8 – 43.5)

n: number; mt: month; BCS: body condition score; kg: kilogram

Table 2 Animal profile used in Chapter 5 Section 5.2.2

Category	Control (n=8)		Clinical (n=6)	
	Median	(Range)	Median	(Range)
Breed (n)	Cross Breed (1)		Cross Breed (1)	
	Jack Russell Terrier (1)		Golden Retriever (2)	
	Rottweiler (1)		Labrador Retriever (1)	
	Staffordshire Bull Terrier (5)		Siberian Husky (2)	
Gender (n)	Entire female (2)		Entire female (1)	
	Entire male (5)		Neutered female (3)	
	Neutered male (1)		Neutered male (2)	
Age (mt)	30.0	(24.0 – 36.0)	63.0	(35.0 – 91.0)
BCS (0/9 - 9/9)	4	(3 – 5)	6	(4 - 7)
Bodyweight (kg)	17.0	(11.0 – 26.0)	33.3	(20.0 – 39.7)

n: number; mt: month; BCS: body condition score; kg: kilogram

Table 3 Animal profile used in Chapter 5 Section 5.2.3

Category	Control (n=6)		Clinical (n=6)	
	Median	(Range)	Median	(Range)
Breed (n)	Jack Russell Terrier (1)		Cross Breed (1)	
	Staffordshire Bull Terrier (5)		Golden Retriever (2)	
			Labrador Retriever (1)	
			Siberian Husky (2)	
Gender (n)	Entire male (6)		Entire female (1)	
			Neutered female (3)	
			Neutered male (2)	
Age (mt)	30.0	(24.0 – 60.0)	63.0	(35.0 – 91.0)
BCS (0/9 - 9/9)	5	(2 – 5)	6	(4 - 7)
Bodyweight (kg)	17.2	(13.0 – 35.0)	33.3	(20.0 – 39.7)

n: number; mt: month; BCS: body condition score; kg: kilogram

Table 4 Animal profile used in Chapter 5 Section 5.2.4

Category	Control (n=1)		Clinical (n=1)	
	Median	(Range)	Median	(Range)
Breed (n)	Jack Russell Terrier (1)		Siberian Husky (1)	
Gender (n)	Entire male (1)		Neutered male (1)	
Age (mt)	36.0	-	35.0	-
BCS (0/9 - 9/9)	5	-	6	-
Bodyweight (kg)	15.0	-	33.2	-

n: number; mt: month; BCS: body condition score; kg: kilogram