THE ROLE OF OBESITY, SYSTEMIC AND SYNOVIAL ADIPOCYTOKINES IN CANINE OSTEOARTHRITIS

by

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ABSTRACT

Osteoarthritis, OA, affects a significant portion of the canine population and is a major source of joint pain and decreased quality of life. Given the lack of disease modifying therapies, the search for factors that influence disease severity and progression continues. As obesity is a common co-morbidity in the canine, weight loss is an important management strategy. In humans, obesity leads to progression in hand OA, indicating that biomechanical changes are not the sole factor in disease progression. While there is evidence for a link between adipokines and human OA, there is no evidence regarding the role of adipokines in canine OA.

In a first set of experiments leptin was quantified in synovial fluid (SF) from healthy dogs and those with OA and compared to body condition score (BCS), radiographic severity of OA, and Liverpool Osteoarthritis in Dogs (LOAD) score.

Synovial fluid was higher in overweight dogs compared to ideal weight dogs and no difference based on the presence of OA was found. No correlation was found between SF leptin and radiographic severity of OA. There was a weak correlation between SF leptin and LOAD.

In a second experiment, SF and serum cytokines were quantified in dogs with OA and in healthy control dogs. Concentrations of SF IL-8, MCP-1 and IL-6 were significantly higher in dogs with OA compared to healthy dogs. There was no significant correlation between serum or SF chemokines/cytokines. Additionally, no correlation was found between any SF cytokine/chemokine and SF leptin.

The final set of experiments involved quantification of SF resistin in healthy dogs and those with OA. Serum and SF resistin was evaluated for correlation with BCS, radiographic severity of OA, and LOAD. There was no difference in serum or SF resistin based on BCS or OA status. There was no correlation between SF resistin and LOAD, or radiographic score. Given these findings along with evidence that resistin is released from monocyte cell lines in people, release of resistin from canine peripheral blood mononuclear cells and adipocytes was investigated. Results of the cell cultures indicate that resistin is released from T cells but not from adipocytes.

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DEDICATION

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CHAPTER 1

INTRODUCTION

Osteoarthritis, OA, is characterized by articular cartilage degeneration, synovitis, and periarticular bony changes [1]. This is secondary to a shift in the balance between destructive and protective enzyme secretion [2]. It occurs commonly in canines and is a major source of pain, impaired mobility, and decreased quality of life [3]. Despite the prevalence of OA, it is often under treated in veterinary medicine. Treatment is often targeted at alleviating pain; however, no current therapies are effective at modulating the underlying disease.

The prevalence of obesity in canines is growing and often occurs simultaneously with OA. While weight loss may be recommended in the management of canine and human OA, the biomechanical and systemic inflammatory effects of weight loss have not been determined in canine patients. Adipocytokines are inflammatory mediators released by white adipose tissue, that may play a role in systemic [4] and synovial inflammation [5]. The main cytokines evaluated in human OA include leptin and resistin.

Leptin is a protein that is released from white adipose tissue [6] and is important in signaling satiety and increasing energy expenditure [7]. Leptin has been found to be positively correlated with body fat content[8] and body condition score [9, 10]. In humans, leptin within the synovial fluid may provide an important link between obesity and OA, as synovial fluid leptin is higher in humans with OA compared to healthy

controls [11]. Additionally, in human OA chondrocytes *in vitro*, leptin stimulated release of matrix metalloproteinases (MMP) -9, -13, and IL-1 β [12].

Resistin is a molecule that is classified as an adipocytokine, as it is released from rodent adipocytes[13]. However, in humans, this pro-inflammatory cytokine is primarily released from monocyte / macrophage cell lines [14]. In two canine studies, serum resistin concentrations were not different in obese compared to ideal weight dogs [15, 16]. Similar to leptin, synovial fluid resistin has been correlated with OA severity in humans [17, 18]. Also, associations have been found among synovial fluid resistin and MMP-1, -3, and IL-6 [19].

This dissertation study had three aims. The first aim was to quantify synovial fluid adipocytokines, particularly leptin, and correlate this to serum leptin and body condition score, BCS, in both healthy control and OA canines. Chapter 3 describes the correlation of synovial fluid leptin with BCS but a lack of correlation with OA status in the canine stifle. Additionally, no correlation was found between radiographic severity of OA, pain and joint dysfunction as measured by Liverpool Osteoarthritis in Dogs (LOAD) score, or synovial fluid IL-1β.

The second aim was to determine the correlation between the synovial fluid adipokines, leptin and resistin, and inflammatory mediators, IL-6, IL-8, MCP-1, and TNF-α. Chapter 5 determined there were elevated levels of synovial fluid IL-6, IL-8, and MCP-1 in the synovial fluid of OA canine stifles compared to healthy stifles; however, no correlation between mediators of inflammation and adipokines existed.

The final goal was to assess the correlation of the reported adipokine, resistin, with BCS and OA status in dogs. Chapter 4 evaluated the lack of serum or synovial fluid

resistin correlation with BCS or OA status. Similar to synovial fluid leptin, no association was found between synovial fluid resistin and LOAD or radiographic score. Additionally, resistin release from stimulated mononuclear cells and adipocytes was investigated to determine if resistin is truly an adipocytokine in dogs. It was found that T cells in culture secrete resistin upon stimulation with Concanavalin A (Con A); however, adipocytes did not release resistin after stimulation with lipopolysaccharide or Con A.

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CHAPTER 2

LITERATURE REVIEW

2.1 Osteoarthritis Pathophysiology

Osteoarthritis, OA, is a major pathologic condition that induces pain, joint dysfunction, and may decrease quality of life in the canine. It is thought to occur in approximately 20% of all canines and is generally secondary to trauma such as joint laxity, joint incongruence, and cyclic trauma to a normal joint [1]. OA is characterized by hyaline articular cartilage degeneration accompanied by bony changes, subchondral bone thickening and osteophyte production, and synovitis [2].

Articular cartilage is the load-bearing surface of diathrodial joints and functions to absorb mechanical shock and evenly distribute load across the joint. It is an avascular tissue [3] made up of a cellular component and an extracellular matrix. The only cell type present within healthy cartilage is the chondrocyte [4], yet these cells represent only about 2-5% of the cartilage tissue volume [1]. The chondrocytes main function is to produce and maintain the extracellular matrix (ECM) [3]. The ECM is composed of a solid organic phase, made up primarily of proteoglycans and collagen and an interstitial fluid phase, which is primarily water [5].

The chondrocyte, despite being only a small portion of articular cartilage, is the key cellular component in maintaining articular cartilage structure and function. There are 3 morphologically distinct types of chondrocytes, Type 1, 2, and 3. The type 1 chondrocytes are the "clonal" cells and are found in the superficial and upper middle zone of cartilage. The type 2 chondrocytes are the secretory type and are found in the middle zone. Type 3 chondrocytes are degenerative cells, that display varying degrees of degeneration, and are found in all zones of articular cartilage [6]. These chondrocytes not only synthesize the ECM but also secrete proteinases (matrix metalloproteinases) that degrade the ECM [7]. The feedback and maintenance of the ECM via chondrocytes is under the control of various growth factors (VEGF, FGF-2, IGF-1) and cytokines (TGF-β1) [8] and through feedback of the ECM receptors, integrins. The integrins are made up of one α and one β subunit and there are 15 α and 8 β subunits that have been identified. The integrins can be ECM protein specific or be more promiscuous and bind multiple ECM proteins. Chondrocytes have been shown to express integrins for type II and type IV collagen, vitronectin, osteopontin, laminin, and fibronectin. Feedback from these receptors determines whether ECM degradation or synthesis will occur [9]. This delicate balance is important in the maintenance of a healthy, well-functioning ECM.

The ECM is the main component of hyaline cartilage responsible for distribution of force over the subchondral bone and it allows for smooth joint motion with minimal friction. The ECM is mainly composed of collagen, particularly type II collagen and proteoglycans, mainly proteoglycan, and hyaluronan.

Collagen forms the structural support of the ECM by stabilizing the matrix and also provides the tensile and shear strength of cartilage[8]. It exists as a monomer

consisting of 3 polypeptide, α chains [1]. While there are 19 different types of collagen identified, the predominant collagen of articular cartilage is type II collagen [1, 10, 11]. Smaller amounts of type VI, IX, X, and XI also can be found in articular cartilage [12, 13]. An important modification of articular cartilage is the ability of collagen to form covalent intrafibrillar and interfibrillar hydroxypyridinium crosslinks [14]. The primary function of collagen II fibrils is to covalently bind proteoglycans into a structural gel that binds water, that allows for many of the biomechanical properties of cartilage [13]. Collagen XI is found at the core of collagen II fibrils and collagen XI covalently binds to collagen II. Based on their locations, both collagen IX and XI are thought to play a role in maintaining collagen II structure during swelling and shrinkage of proteoglycans [1, 11].

The proteoglycans allow for the viscoelastic properties of articular cartilage[15, 16] by altering interstitial fluid flow under load [16]. Proteoglycan is comprised of a protein core attached to numerous glycosaminoglycan (GAG) chains [4, 7, 15, 17, 18]. Proteoglycans that have been well categorized are aggrecan, decorin, biglycan, and fibromodulin, with aggrecan being the largest and most abundant [7]. The core protein of aggrecan is comprised of 3 distinct globular domains. The G1 domain, located on the N-terminal, allows for binding to a link protein that ultimately results in aggrecan binding to hyaluronan and aggrecan aggregation [19]. The G2 domain is adjacent to G1 although the function of this domain is not well understood but is likely important in link protein binding [20]. The G3 domain, on the carboxyterminal, has several subdomains, such as epidermal growth factor-like and lecithin-like subdomains, that allow for interaction with smaller ECM molecules [19]. Situated between the G2 and G3 domain are 2 domains, that allow for binding of the 2 main GAGs, chondroitin sulfate and keratan sulfate, with

the chondroitin sulfate domain being the largest of the 2 [20]. These GAGs are anionic molecules that result in a large osmotic gradient that draws water into the cartilage to allow for absorption of compressive forces. The expansile properties imparted by GAGs are balanced by the tension within collagen fibrils.

There are several smaller proteoglycans in articular cartilage, notably biglycan and decorin. Structurally, these molecules consist of a small, leucine rich, core protein that binds with 1-2 dermatan sulfate or chondroitin sulfate chains [3]. Biglycan is found mainly in pericellular sites and on the chondrocyte cell surface and therefore may play an important role in morphogenesis and differentiation [21]. Decorin is found mostly in the superficial zone of articular cartilage [3] and is important in regulation of matrix organization, cellular adhesion, migration, and proliferation [21].

Under light microscopy articular cartilage, appears in 4 distinct zones, the superficial (tangential), the intermediate (transitional), deep (radial) and the calcified layer [11]. The superficial zone, which is in contact with synovial fluid, has a low proteoglycan content and collagen fibrils that are parallel to the cartilage surface [14]. The chondrocytes are flattened and run parallel to the cartilage surface and can synthesize and secrete a protein called superficial zone protein or lubricin. Lubricin, also secreted by synoviocytes, is important in allowing synovial fluid to create a near frictionless surface. This area of articular cartilage has the greatest tensile strength [22]. Unlike the superficial zone fibrils, the fibrils within the remaining deeper zones are all arranged randomly. The next zone following the superficial zone is the transitional or intermediate zone, which comprises about 40-60% of the total cartilage. It is a less cellular zone with spheroidal cells, increased proteoglycan content and a dense, thick fibril arrangement. While it does

function as the name implies as a transition from superficial to deep zones, it is important as it is the first line of defense against compressive forces. The deep zone, which provides the greatest resistance to compression [7], contains a low density of cells aligned in columns perpendicular to the joint. These cells are the most metabolically active compared to chondrocytes in other zones [23]. Despite having low collagen concentrations, it contains fibrils of the largest diameter and a large aggrecan content [4, 22]. Lastly, the calcified layer contains, smaller spheroidal cells, and is partially calcified [4]. It provides surface attachment to subchondral bone [1] and provides a buffer between the mechanical properties of the articular cartilage and underlying subchondral bone [22].

The joint is enclosed entirely by the joint capsule, which is comprised of the synovium and an outer fibrous tissue [1]. The synovial membrane plays an important role in maintenance of the joint, contributes to the pathology of OA [24] and may be adversely affected in joint disease [25] . The synovial membrane is the site of synovial fluid production, which is crucial for chondrocyte nutrition and joint lubrication [24]. The synovial membrane consists of two layers, the intima and the subintima, with a small amount of hyaluronan between layers. The intima is relatively acellular but consists of two types of synoviocytes, type A, which is of macrophage or monocyte cell lines and type B, which is a fibroblast. The subintima is composed of blood and lymphatic vessels [26] and loose connective tissue and merges with the outer layer of the joint capsule [24].

The synoviocytes are important in producing synovial fluid for cartilage lubrication, chondrocyte nutrition, and control of synovial fluid volume through the production of hyaluronic acid [24, 26]. They may also play an important role in joint

pathology by increasing joint friction, regulation of inflammation, and fibrosis [24] and regulation of synovial immune function. An extensive discussion of synovial receptor localization, structure, and function can be found in Appendix A.

2.2 Canine Osteoarthritis

Osteoarthritis occurs when there is break down or degeneration of articular cartilage. Unlike in humans, canine OA is almost exclusively secondary to joint trauma, joint laxity, or repetitive high impact loading [12]. Chondrocytes, as previously discussed, secrete numerous proteinases, cytokines, and prostaglandins that result in cartilage destruction. These degradative substances are normally balanced by anti-inflammatory cytokines and growth factors. When there is a shift in the balance between the destructive and protective enzyme secretion, OA will develop [27].

While OA affects every structure that comprises the joint, the main changes associated with OA consist of articular cartilage loss, remodeling of subchondral bone, and production of osteophytes [4]. Due to the lack of blood supply to the cartilage, this tissue is affected early in the progression of the disease. Morphologic changes that occur in OA cartilage include changes in contour, as the cartilage becomes fibrillated, develops a roughened surface, and displays ulceration and cracking. Additionally, there are changes in thickness, with progressive thinning, as well as changes in color (changes from yellowish to a brownish color due to appearance of vessels within the subchondral bone). Often there is a change in consistency as the cartilage becomes softened and chondromalacia can develop [27]. Biochemical changes are characterized by increased

cartilage hydration [4, 14], disorganization of the collagen network [14], decrease in aggrecan and proteoglycan, and structural changes to the glycosaminoglycan chains [4, 14]. Biomechanically, the tensile stiffness and ion induced stress relaxation are both decreased [14]. Because the turnover of adult cartilage, especially the collagen framework, is extremely slow, there is little capacity for chondrocytes to recapitulate the overall cartilaginous architecture once there is damage [10].

Changes to the components of articular cartilage, as described above, are mediated primarily by cytokine release from both chondrocytes and synoviocytes. During OA, there is a major shift to a catabolic and pro-inflammatory state occurs. Synovitis, possibly due to mechanical injury from joint instability or incongruity and intra-articular shear stress, is the root cause of cytokine release from the synovium [28]. The chondrocytes are stimulated to release inflammatory mediators due to both biomechanical derangements, such as joint malalignment, or due to inflammatory mediator release from the synovium [25]. Cells in both tissue types are capable of releasing IL-1β, TNF-α, aggrecanase-2 (ADAMTS-5), nitric oxide, (NO) and matrix metalloproteinases [25] especially the collagenase MMP-1, MMP- 8, MMP-13 and MMP-3, which is specific for type II collagen [11]. Release of IL-1β and TNF-α induce release of other cytokines such as IL-8, IL-6, prostaglandin E_2 (PGE_2), and NO. NO and reactive oxygen species induce chondrocyte apoptosis and inhibit proteoglycan and collagen synthesis [25], while IL-6 has been shown to promote the production of tissue inhibitor of metalloproteinases (TIMPs) [28].

While synovitis is classically described in numerous arthropathies, there are actually 4 subtypes of synoviopathies present in OA. These subtypes include

hyperplastic, inflammatory, fibrotic, and detritus-rich, with both the fibrotic and detritus-rich subtypes having significant fibrosis [24]. Initial changes include hyperplasia and synovial membrane thickening. Additionally, there is an increase in vascularity and mononuclear cell invasion. Eventually fibrosis develops within the synovium, which is frequently more pronounced on the medial aspect of the canine stifle. These changes are thought to be due to phagocytosis of proteoglycans and collagen fragments in the synovial fluid by synovial macrophages. This results in an inflammatory reaction within the synovium, which perpetuates cartilage damage and further inflammation [1]. Development of synovial fibrosis is thought to be under the control of both Smad-dependent and independent TGF-β pathways. Also, $PGF_{2\alpha}$ is elevated in OA synovial fluid and likely contributes to synovial fibrosis. These effects are blocked by intra-articular administration of hyaluronan through an unknown mechanism [24]. Overall, synovial fibrosis results in joint stiffness, decreased mobility, and contributes to pain in OA.

Osteoarthritis also affects subchondral bone. Inflammation and changes in biomechanics may result in subchondral sclerosis, microfractures within superficial bone trabeculae, development of bone cysts, and production of osteophytes. These osteophytes are generally found around the periphery of the joint but may also invade the joint capsule [27].

Several studies have reported changes in biomarkers and cytokines within the synovial fluid of canine OA joints compared to healthy joints. Synovial fluid concentrations of the GAG, keratan sulfate, have been evaluated in several canine studies and have reported conflicting results. Some studies report elevated concentrations in OA

joints compared to controls, some report no difference, and others report decreased keratan sulfate in OA joints [7]. The C-propeptide of type II collagen has also been found to be elevated in experimentally induced OA compared to the unaffected joint [29]. Similar findings have been reported in human OA synovial fluid [7]. Additionally, MMP-3 and TIMP-1 have been shown to be elevated in naturally occurring OA. Finally, several cytokines, such as PGE_2, TNF-α, and IL-6, have been found to be at higher concentrations in canine OA synovial fluid. While TNF-α is elevated in OA synovial fluid, the increase is to a much lesser extent than IL-6 [30]. Synovial fluid concentrations of PGE_2 are positively correlated with lameness and negatively correlated with peak vertical force as measured by force plate [31]. IL-6 has also been positively correlated to proteoglycan synthesis in experimental canine OA [30]. Despite the current literature, further studies on biomarkers, cytokines, and adipokines in canine OA synovial fluid are warranted.

2.3 Canine Obesity

Canine obesity is also frequently seen in veterinary patients and may contribute to other systemic pathologies. Obesity in canines has been previously defined as a body weight that is at least 15% greater than ideal body weight [32, 33]. More recent studies report that approximately 43 – 59% of canines fit this definition of obesity [34, 35]. In one study, obese dogs were reported by caregivers to have a lower quality of life than their lean counterparts and quality of life improved when weight loss was achieved [36]. There

is also significant evidence that obesity is correlated with other pathologic diseases in both humans and canines

In humans, obesity has been linked to hypertension, increased plasma insulin concentrations and insulin resistance, diabetes mellitus, hyperlipidemia [37], and systemic, chronic low-grade inflammation, as evidenced by increased TNF-α, IL-6, C-reactive protein, and haptoglobin [38]. In mice a phenotypic switch from M2 to M1 macrophages in adipose tissue is seen with obesity, however, human adipose tissue macrophages display both phenotypes. These macrophages have been shown to release not only anti-inflammatory cytokines such as IL-10 and IL-1Ra but also can release TNF-α, Il-1β, Il-6 and MCP-1 [39].

In canines, total cholesterol [40-42], triglycerides [40, 43], low density lipoprotein, insulin like growth factor, glucose [43], and insulin are higher in obese compared to optimal weight dogs [43, 44]. Also, cholesterol, triglycerides [43], systolic blood pressure and insulin reportedly decreased after weight loss [41]. Still, all values were within the reference interval and thus, the clinical significance is currently unknown. Additionally, the link between obesity and low-grade inflammation in the canine remains controversial. In one study, serum levels of C-reactive protein (CRP) in obese canines were lower when compared to normal weight controls [44]. In another study evaluating weight loss in obese dogs, CRP, TNF-α, and haptoglobin [45] were found to decrease. While yet another study reported no change in CRP with weight loss [41].Further, monocyte chemoattractant protein-1 (MCP-1) was also found to decrease with weight loss in dogs [46]. It is worth noting that both IL-6 and MCP-1 mRNA expression has been also demonstrated in canine adipose tissue [47]. Finally, owner

perceived quality of life was found to be improved in optimal weight dogs compared to obese canines [48] and dogs with lower caloric intake manifested by lower body weight had a longer median lifespan [49].

The gold standard for the evaluation of body composition is typically achieved through cadaveric analysis, however this is not useful in living animals, particularly in clinical trials [50]. Because of this lack of utility in living animals, dual-energy X-ray absoptiometry, DEXA, is frequently employed. DEXA is essentially an enhanced form of X-ray analysis that utilizes 2 distinct photons of energy, 70 and 140 kVp. Due to the fact that bone mineral, lipid, and lean tissue impede these beams of energy differently, a computer derived algorithm is able to calculate the amount of each tissue type in each pixel scanned [51]. A limitation of this technique is that there is an assumption that lean body mass is uniformly hydrated. An additional technique that has been reported in canine populations is deuterium dilution. After administration of isotope (deuterium) labelled water to a subject, deuterium is sampled in saliva, urine or blood and measured via isotope ratio mass spectroscopy [50]. While both of these techniques can be used in the clinical or research setting, cost and availability limit their routine use. Therefore, techniques such as morphometric measures and body condition score, were developed as clinically useful tools to classify obesity in veterinary medicine. Body condition score, BCS, essentially involves subjective evaluation of physical characteristics (i.e. presence of a waist and abdominal tuck, fat covering the ribs) and has been positively correlated to DEXA in dogs [52]. BCS can be measured on a 5-point scale with a scores of 1-2 being underweight, 3 being ideal, and 4-5 are overweight. It can be measured on a similar 9-point scale, where 1-3 are underweight, 4-5 is ideal, and >5 correlates with varying

degrees of obesity. Percentage body fat for ideal BCS averages 20% and ranges from 15 to 25% of body weight. Each 1-point change from ideal represents an increase or decrease of 10% change in body fat using the 5-point scale and 5% increase or decrease using the 9-point scale. Finally, several techniques that employ objective measures of trunk and limb lengths have been developed for use in dogs, and have shown good correlation with DEXA [53].

2.4 Obesity and Osteoarthritis

In human patients, obesity is associated with an increased incidence of and progression of OA [54, 55]. Further, in patients where OA is confined to a single joint, obesity increases the risk for the development of OA in additional joints [56]. The link between obesity and OA can be explained by the theory that obesity results in greater repetitive axial loading of the joint. In human patients, BMI in combination with joint mal-alignment are factors that appear to impact OA severity [57, 58], indicating that increased load bearing with increased BMI may play a role in OA progression. While changes in kinematics and joint loading with obesity may be an important factor in the development of OA in load-bearing joints, changes in biomechanics do not explain the link between OA and non-load bearing joints (i.e. hand and shoulder).

The link between OA and obesity in dogs is not as thoroughly researched, however several studies in dogs demonstrate the negative impact of obesity on OA. In a study by Adams et al, obesity quadrupled the risk for development of cranial cruciate ligament rupture. In a series of studies involving 48 Labrador Retrievers that consisted of

24 paired littermates divided into 2 groups and followed for 13 years, the effect of dietary restriction on the incidence and severity of OA was examined. All dogs within these studies were paired based on size, age, and sex. Each dog in a pair was then randomly assigned to receive a control or calorie restricted diet. The dog within in the control group was fed ad libitum and the dog in the restricted group was fed 75% of the amount its paired control dog ate the previous day. The dogs were then monitored for OA in the hip, shoulder, and elbow. The dogs within the restricted fed group lived approximately 1.8 years longer than the control fed group and had a lower hazard of death from musculoskeletal disorders [49]. Additionally the dogs within the control fed group had a higher prevalence of shoulder OA [59] and the severity was significantly higher at 6 and 8 years old in this group compared to the restricted fed group [60]. In the elbow, the severity was significantly different between groups, with controls dogs having more severe OA [59, 61]. The most profound differences were found in both the incidence and severity of coxofemoral OA. Significantly more dogs within the control fed group developed OA, which was more severe in this group [59]. Additionally, dogs within the control group developed radiographic evidence of OA earlier life, at median age of 6 years old compared to 12 years old in the restricted fed group [62]. In regards to lameness secondary to OA, several studies have noted subjective improvement in lameness scores after obese dogs lost weight [63, 64]. Despite this link between OA and obesity in dogs, there are no current studies evaluating the mechanism for this association.

2.5 Canine Adipocytokines

There are two main types of adipose tissue, white and brown, with white being the main type of adipose tissue in adult mammals [65]. White adipose tissue, WAT, is comprised of pre-adipocytes, adipocytes, and the stomovascular fraction, which is made of macrophages, endothelial cells, fibroblasts, and leukocytes, and is important in regulation of metabolism and inflammation [66]. As macrophages are a large component of the stromovascular portion of WAT, pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 are released from WAT, particularly the macrophage component [65, 67]. Additionally, there are molecules released directly from the adipocytes, adipocytokines, that regulate both inflammation and metabolism [65-67]. The most commonly evaluated adipokines include leptin, adiponectin, visfatin, and resistin.

2.5.1 Leptin

Leptin is a 16kDa protein that arises from the Ob gene and is released from WAT in both humans and canines [68]. Leptin binds to the Ob-R, which is classified as a Type I cytokine receptor [69]. While there are at least 6 isoforms of the ObR, the ObR long (ObRb) and ObR short (Oba) forms are the 2 most studied isoforms. ObRb is primarily found in the hypothalamus and the ObRa is the soluble form and is found circulating in blood. Signaling through the long form ObRb is thought to be responsible for the majority of the effects. ObRb signaling is primarily via the tyrosine kinase receptor, Janus kinase 2 (JAK-2), which results in activation of STAT1 and STAT3. Additionally, activation of ObRb results in insulin receptor substrate-1 phosphorylation and activation of phosphotidylinositol- 3 kinase (PI3K) and Akt [70]. Additionally, the leptin receptor

signals similarly to the signaling of the IL-6 family; however, unlike IL-6 receptors, ObR does not require gp130 to induce receptor activation [71]. Thus, signaling through this JAK – STAT pathway results in nuclear translocation of STAT and gene transcription of cytokines and growth factors. Both secretory IL-6 and IL-10, which are released from human B cells after stimulation with leptin have been shown to be mediated through the ObR and JAK-STAT3 pathway [72]. Interestingly, IL-6 release from murine microglia is mediated by the IRS-1, PI3K, Akt cascade [73].

In human WAT, Ob gene expression is increased in obese subjects compared to lean cohorts and gene expression is positively correlated to BMI [74]. Leptin is released in proportion to white adipose tissue mass by adipocytes. The purpose of leptin release is to signal satiety and prevent weight gain. Leptin specifically acts within the satiety center of the hypothalamus to decrease food intake [75]. Within the hypothalamus, it specifically activates pre-opiomelanocortin (POMC) neurons within the arcuate nucleus. While the POMC neurons play a major role in leptin function, they are not the sole mediators of leptin induced weight gain as complete loss of leptin receptors causes more significant weight gain than blockade of POMC neurons [76].

In dogs, plasma leptin has been studied and found to correlate well with body fat content as determined by deuterium dilution [77]. Additionally, leptin levels directly correlate with body condition score, [77-79] being higher in dogs with higher body condition scores [78]. In another canine study involving experimentally-induced obesity, leptin was measured prior to the development of obesity and throughout the period of weight gain. In these dogs, plasma leptin increased with weight gain [80]. Plasma leptin has also been shown to decrease in obese dogs after a period of weight loss [81].

Leptin has been evaluated in several disease and pharmacological states in the dog. Dexamethasone (0.1mg kg^{-1}) has been shown to enhance the increase in plasma leptin due to feeding [82]; however daily prednisolone had no effect on leptin concentration [83]. In another study, methylprednisone had a dose dependent effect on plasma leptin concentration. At a dose of 1 mg kg^{-1}, an increase in leptin was seen. With 5mg kg^{-1}, a biphasic response was noted. There was an initial decrease followed by a rise in plasma concentration, while 10mg kg^{-1} resulted in a decrease in plasma leptin concentration [84]. Typically, an increase in abdominal fat mass is seen with naturally occurring pituitary dependent hyperadrenocorticism. Additionally, in this canine population, plasma leptin and insulin concentrations were elevated compared to healthy dogs but no difference in resistin, adiponectin, TNFα, IL-1β, IL-6, IL-10 or IL-18 was seen among the groups [85]. In canine hypothyroidism, higher concentrations of plasma leptin are documented [86]. Both benign and malignant masses have shown to express leptin, ObR, and adiponectin, with ObR expression being higher in the malignant neoplasms [87]. While there are a limited number of clinical studies regarding leptin concentrations in various states in the canine, there are no studies regarding leptin in canine OA.

2.5.2 Adiponectin

Adiponectin is another adipokine that is found in human, rodent, feline, and canine plasma. Canine adiponectin shows approximately 80-91% homology to adiponectin molecules in other species [88]. It exists in canine plasma as multimers, both a low and

high molecular weight complex. The low molecular weight complex is 180kD, while the high molecular weight complex is comprised of 4 to 6 multimers and weighs >360kD [89]. In dogs, adiponectin mRNA has been detected only WAT [47].

Adiponectin, in humans [90, 91], horses [92], and cats [93] is higher in lean compared to obese subjects and negatively correlated with body fat mass. Additionally, there is a negative correlation between body fat mass and serum adiponectin [92, 93]. Increases in plasma adiponectin are seen during human weight loss [94]. In cats and humans, adiponectin is thought to increase insulin sensitivity. As lower concentrations of adiponectin are seen in obesity, it may play a role in insulin resistance and lower glucose tolerance [95, 96]. Additionally, higher adiponectin concentrations are associated with a lower risk of development of type II diabetes mellitus [97].

There are several studies evaluating serum or plasma concentration of canine adiponectin; however conflicting results have been reported. In at least two separate studies, adiponectin was found to be higher in lean versus obese dogs and was negatively correlated with BCS and serum leptin concentration [79, 88]. While these studies are consistent with findings in other species, several other studies have reported contradictory findings. These studies have reported no association between plasma adiponectin and adiposity [98, 99]. Unlike in other species, adiponectin in dogs does not increase in obese dogs during weight loss [45, 46]. Additionally, there is no correlation between plasma adiponectin and insulin resistance or pancreatic β-cell function [98].

Adiponectin, in humans, is the main anti-inflammatory adipocytokine. A study in obese women found that adiponectin was negatively correlated with CRP and IL-6 levels

[100]. In both human macrophages [101] and endothelial cells [102], adiponectin induced production of anti-inflammatory proteins in response to TNF-α. In contrast, the anti-inflammatory role of adiponectin in dogs is less well researched. In a single canine study, the investigators reported that adiponectin decreased in response to LPS infusion suggesting a negative correlation with LPS [103]. Overall, the evidence regarding canine adiponectin in inflammation and disease modulation is limited and warrants further investigation.

2.5.3 Resistin

Resistin is a cysteine – rich molecule that was originally discovered in the mouse and is released during adipocyte differentiation in rodents [104]. However, in humans, resistin is released mainly by macrophages with a small amount being released by WAT [105]. Even in WAT, secretion of resistin is predominantly from monocyte / macrophage cell lines [106]. In another study, resistin mRNA expression was not detected in human adipocytes [107]. Early after the discovery of resistin, in rodent models, it was thought to be correlated with obesity and insulin resistance [104], however more recent evidence provides conflicting results regarding the role of resistin in obesity. In obese rodents, some studies have shown that serum resistin is increased compared to lean rodents [104, 108], while others report a decrease in resistin gene and mRNA expression in obese rodents [42, 108-110]. In humans, increased serum resistin levels have been reported in obese [111, 112] and morbidly obese women [112], as well as women with gestational diabetes [113] compared to healthy controls. However, other studies report that there is

no difference in serum resistin in obese [91] or diabetic [114] subjects compared to lean and healthy humans, respectively. Additionally, to the author's knowledge only one study reports correlation between resistin and body mass index, BMI [112, 115]. More studies provide evidence to support a lack of association between resistin and BMI [113, 115], percent body fat [114], insulin resistance [113], or glucose tolerance [115].

In canines, the role of resistin is less well known and studied. One study evaluating serum resistin in lean (BCS4-5/9) versus obese (BCS 7-9/9) canines, found no significant difference between resistin based on weight class [116, 117]. Additionally, no differences were detected in serum resistin in canines with pituitary-dependent hyperadrenocorticism [85] or diabetes mellitus [118] compared to healthy control. However, higher serum resistin concentrations have been found in canines with acute pancreatitis [119] and those with diabetic ketoacidosis [117] versus healthy canines. Based on these findings, further research into the role of resistin in canine health and disease is warranted, particularly in the setting of inflammation.

2.6 Adipocytokines and Osteoarthritis

While there is a clear link between OA and obesity, the mechanisms are less well understood in humans and not evaluated in canines. Given the association of hand OA and obesity in humans, the underlying mechanism is unlikely to be purely biomechanical in nature [120]. Systemic and local adipocytokines are thought to play a role in the link between OA and obesity. Evidence for the role of adipokines in OA have been reported within the human literature; however, no canine studies exist.

In a mouse model of obesity, knock out of leptin or leptin signaling resulted in development of extreme obesity; however, none of these animals developed OA [121]. This rodent study would appear to implicate leptin in the development of OA. The adverse effects of leptin and obesity on OA have also been reported in humans. Fowler-Brown et al reported that the adverse effects of BMI on OA were at least partially mediated by serum leptin concentrations. It has also been shown to be positively associated with knee OA in people. Additionally, in middle aged women, higher serum leptin concentrations predict greater odds of the development of knee OA [122]. Serum leptin has been correlated with cartilage volume loss, assessed by MRI, in human OA knees [123] and with radiographic hip joint space narrowing [124]. Surprisingly, no evidence exists that links serum leptin concentrations to histologic markers of cartilage damage or synovial inflammation [125]. Despite the evidence for serum leptin in OA, there is at least one conflicting study which found no association between serum leptin and development or progression of OA [126].

Given the conflicting evidence regarding the role of systemic leptin in OA, evaluation of local leptin concentrations is still warranted. Synovial fluid (SF) leptin has been found to be correlated to BMI [127, 128] but waist circumference is reportedly a better predictor of SF leptin [129]. SF leptin has also been shown to exceed serum leptin [128, 130] and is higher in SF from OA people compared to healthy controls [131]. SF leptin was higher in people with severe disease than those with mild to moderate OA. It was previously thought that leptin was released into the joint from the infra-patellar fat pad but leptin release has been demonstrated from human OA synovium, cartilage, infra-patellar fat pad, meniscus, and osteophytes [130].

Given that there are significant concentrations of local articular leptin, the effects on cartilage have been evaluated *in vitro*. Human OA chondrocytes cultured with leptin for 7 days released elevated concentrations of MMP-9, MMP-13, and IL-1β [128] and downregulation of leptin resulted in decreased MMP-13 expression [132]. At supraphysiologic doses (0.1μg kg^{-1}; 10μg kg^{-1}), leptin enhanced production of nitric oxide (NO), prostaglandin E_2 (PGE_2), cycloxygenase-2 (COX-2), IL-6, IL-8, [133] and low levels of collagenase. The collagenase release was shown to be synergistic with IL-1β and TNF-α [134]. The same concentrations of leptin, in another study, resulted in MMP-1, MMP-3, and MMP-13 release. These in vitro results support the finding that SF leptin is positively correlated with MMP-1 and MMP-3 release [135].

Despite the fact that resistin is less well researched in human OA, there are several studies evaluating its role in human OA but none in canine OA. Higher serum resistin concentrations have been reported in patients with knee [136] and hand [137] OA compared to healthy controls. Not only has serum resistin been identified as a risk factor for the development of hand OA, it also has been associated with IL-17 [138] and histological markers of cartilage damage [125]. However, there has been no association between serum resistin and serum C-reactive protein [139].

As OA can represent a localized disease, local resistin concentrations and effects have been evaluated. Similar to serum resistin, SF resistin does not appear to be correlated with body mass index [139]. Further, SF resistin concentrations tend to be lower than serum resistin [136, 139] and it has been correlated to clinical severity of OA [136, 140] and pain score. Additional, associations have been found between SF resistin and cartilage damage [136], SF IL-6, SF MMP-1, and SF MMP-3 [139]. When

administered intra-articularly to mice, resistin promoted leukocyte infiltration and synovial hyperplasia [141]. With the lack of data regarding resistin in canine OA, further studies on this adipokine are indicated.

Several studies in human OA have demonstrated a role for the adiponectin in disease pathophysiology. In one study, serum adiponectin was observed to be inversely correlated with cartilage loss as evaluated by MRI [123]. Also, serum adiponectin was determined to be lower in patients with progression of hand OA and higher serum adiponectin decreased the risk of disease progression [120]. Finally, lower concentrations of serum adiponectin have been associated with less severe OA grade [142]. In contrast to these findings, adiponectin has been positively correlated with cartilage oligomeric protein, MMP-3 [143] and radiographic severity [144].

Adiponectin has been found in SF, but at lower concentrations than in serum [145]. Similar to systemic adiponectin, SF adiponectin concentrations are inversely correlated with OA grade [142]. However, SF adiponectin has been positively correlated with degenerative fragments of aggrecan [146]. Both adiponectin receptor 1 ($AdipoR_1$) and 2 ($AdipoR_2$) have been shown to be expressed in normal synovial tissue [145]. Interestingly in one study, $AdipoR_1$ or $AdipoR_2$ was absent in non-lesional cartilage but detected in lesional OA cartilage [147]. Similarly, adiponectin expression has been found in OA cartilage but not normal cartilage. In an *in vitro* cartilage culture, a positive correlation between adiponectin mRNA and mRNA of PGE2 and MMP-13 was demonstrated [148]. Adiponectin release has also been associated with NO, IL-6, and MMP-3 [143]. Chondrocytes cultured with adiponectin have enhanced production of NO, IL-6, MMP-1, MMP-3 [143], MMP-13, PGE2 [148], and VCAM-1 [149]. Finally,

culture of synovial fibroblasts with adiponectin induced IL-6 production. While adiponectin is thought to classically be an anti-inflammatory adipocytokine, its function in joint inflammation is unclear and further research is warranted in both human and canines.

2.7 References

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CHAPTER 3

CORRELATION OF SERUM AND SYNOVIAL LEPTIN CONCENTRATIONS WITH BODY CONDITION SCORES IN HEALTHY AND OSTEOARTHRITIC DOGS

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3.1 Abstract

Objective: To evaluate the relationship between serum and synovial fluid (SF) leptin and body condition score (BCS) in healthy and osteoarthritic dogs.

Study Design: Controlled, prospective, clinical study.

Animals: Nineteen healthy dogs and 29 dogs with osteoarthritis (OA) secondary to cranial cruciate ligament injury.

Methods – Synovial fluid was obtained from the femorotibial joint under sedation (healthy dogs) or during surgery (OA dogs). Serum and SF leptin and interleukin (IL)-1β concentrations were measured via enzyme-linked immunosorbent assay. Dogs were classified as optimal weight (BCS 4-5/9) or overweight (BCS >5/9). Radiographs were scored for OA severity by a radiologist. Owners were required to complete the Liverpool Osteoarthritis in Dogs (LOAD) questionnaire.

Results – Mean (± SD) SF leptin (4.09 ± 4 ng/mL) was significantly lower than serum leptin (6.88 ± 5.52 ng/mL, $P < 0.0001$). Synovial fluid leptin was higher in overweight (5.28 ± 4.21) than in optimal body weight dogs (1.54 ± 1.72 ng/mL, $P <0.0001$). Serum ($P < 0.001$) and SF leptin ($P =0.004$) concentrations were associated with BCS. Concentration of SF leptin did not differ between healthy (2.4 ± 2.04 ng/mL) and OA (4.9 ± 4.3 ng/mL, $P = 0.25$) dogs. Synovial fluid leptin and LOAD scores were weakly associated ($P=0.03$). No association was detected between SF leptin and radiographic score or IL-1β ($P = 0.73$).

Conclusion - Serum and SF leptin correlated with BCS in this population. Synovial fluid leptin was weakly associated with LOAD scores but not with radiographic severity of OA or IL-1β.

Clinical Significance - Serum and SF leptin concentrations do not predict radiographic severity of canine OA but contribute to joint pain and dysfunction.

3.2 Introduction

Osteoarthritis, OA, is a common morbidity in small animal practice and it is a major source of pain, dysfunction and decreased quality of life in many canine patients. Therapeutic management recommendations often involve a combination of diet, anti-inflammatory drugs, physical therapy, nutraceuticals, and weight control [1]. Weight loss is an important management technique, given that obesity is a very prevalent (43-65%) co-morbidity [2-4]. However, the evidence regarding the effect of weight loss and obesity on OA in dogs is limited. Canine caregivers perceive that obese pets are generally sicker and less mobile, leading to a decreased quality of life [5]. Two studies in dogs report that weight loss in overweight and obese dogs improves lameness both subjectively [6,7] and objectively [7].

In humans, obesity has been shown to be a risk factor for the development of OA [8]. Additionally, body mass index, BMI, has been associated with the incidence and development of knee osteoarthritis, in a dose responsive manner [9,10]. Humans with knee osteoarthritis, also, report more pain associated with knee OA than patients with a normal BMI [11]. While the link between knee OA and obesity is explained by increased

load on the arthritic joint, biomechanics do not explain the link between BMI and hand OA [8-12].

Adipocytokines are mediators released from white adipose tissue, that may play a role in the link between obesity and osteoarthritis. Leptin, a pro-inflammatory adipocytokine is important in appetite regulation and energy homeostasis [13]. It has been correlated with body fat mass in both humans and canines and body condition score, BCS, in canines [13-14]. Additionally, in humans, higher levels of serum leptin have been found in OA patients compared to healthy controls [15]. In one study in humans, plasma leptin has been associated with systemic and synovial biomarkers of inflammation and was correlated with the radiographic presence and progression of knee OA [16]. Leptin has also been found in human synovial fluid and was positively correlated to serum leptin and BMI [17-18]. Patients with higher serum and synovial fluid leptin levels reported higher pain scores than those with lower leptin concentrations [17-18]. In OA chondrocytes, leptin expression is up-regulated and degree of staining via immunohistochemistry is positively correlated with OA score [19].

Currently there are no studies evaluating synovial fluid for the presence of leptin in the dog. Additionally, the role of synovial fluid and serum leptin in severity of OA or the inflammation and pain associated with canine OA is unknown. The primary objective was to quantify leptin in both healthy and OA canine synovial fluid and to examine the correlation between synovial leptin and measures of body condition scores. The secondary objective was to evaluate the association between synovial and serum leptin and synovial inflammation, radiographic severity, and pain in the OA canine.

3.3 Materials and Methods

3.3.1 Animals - Forty-eight client-owned dogs, 19 healthy control (non-OA) and 29 with OA secondary to cranial cruciate ligament (CCL) injury, were enrolled in the study. The non-OA control group was deemed healthy on the basis of physical examination, orthopedic examination, and routine hematology. Any dog with a history of lameness or pain on palpation of any joint was eliminated from the control group. Dogs in the OA group were deemed healthy, other than CCL disease, based on physical examination, complete orthopedic examination, and hematology. Dogs were excluded from the OA group if there was evidence of lameness, OA, or pain on joint palpation in any joint other than the femorotibial joint. Any dog was excluded from the study if evidence of systemic illness, other than orthopedic disease, was present. Additionally, dogs less than 8kg were excluded due to the inability to collect adequate volumes of synovial fluid. Signed informed owner consent was obtained and all procedures were approved by the University of Georgia Institutional Animal Care and Use Committee.

3.3.2 Morphometric Measures – Body condition score was determined on a standard 9-point scale (Nestle Purina, St. Louis, MO, US) by 3 individual observers, who were unaware of the other raters' scores. For analysis of leptin concentrations by group, dogs were placed into 2 categories based on BCS, optimal weight (BCS 4-5) and overweight (BCS>5). Additionally, thorax, pelvis, and limbs were measured and used to calculate body fat mass (g) and body fat %, as previously described [20].

3.3.3 Radiographic Scoring – All dogs within the OA group underwent pre-operative radiographs of the effected stifle. Standard mediolateral and craniocaudal radiographs

were obtained and scored by a radiologist (CG). Each set of radiographs was scored using a previously described scoring system (Appendix 1) [21].

3.3.4 Sample Collection and Assays - Upon completion of both physical and orthopedic examinations, dogs within the control group were sedated with dexmedetomidine (5mcg/kg) intravenously. Blood was collected via standard jugular venipuncture. A single femorotibial joint was arbitrarily selected for arthrocentesis. The skin over the joint was shaved and aseptically prepared and synovial fluid was obtained through routine arthrocentesis. Joint lavage for the purpose of sample collection was not performed in any dog. For dogs in the OA group, jugular venipuncture was performed after induction of anesthesia but prior to transfer to the operating theater. Synovial fluid was obtained just prior to arthrotomy in an aseptic manner. All dogs in both groups were fasted for 8 hours prior to sample collection. Serum and synovial fluid were placed on ice immediately after collection and then centrifuged at 4°C at 4000 x g to separate cells and debris. Samples that were clear to yellowish in color were stored for later processing. All samples were aliquoted into 250µL aliquots to minimize freeze-thaw cycles and stored at -80°C until analysis. Serum and SF leptin concentration was determined via a canine leptin ELISA (EMD Millipore, Darmstadt, Germany). IL-1β was determined in serum and synovial fluid with a canine specific IL-1β ELISA (ThermoFischer Scientific, Waltham, Ma, USA). Both ELISAs were completed according to manufacturer's instructions. Optical density was measured with a plate reader (Biotek Synergy 4, BioTek Instruments, Inc, Winooski, VT, USA) and associated software (Gen 5, BioTek Instruments, inc, Winooski, VT, USA). Optical density was utilized to calculate leptin and IL-1β concentrations against a standard concentration curve.

3.3.5 Client Questionnaires - All owners were required to complete two separate surveys, a diet history and the Liverpool Osteoarthritis in Dogs, LOAD. The diet history (Appendix 2) included information about type, quantity, and frequency of diet and feeding. It also included details on any nutraceuticals or dietary supplements that each dog received. This was utilized to determine the amount of Ω-3 and Ω-6 each dog consumed on a dry matter basis. Dietary Ω-3 and Ω-6 was determined from published documents from the dog food manufacturer. If no documentation was available, each individual manufacturer was contacted.

The LOAD is a clinical metrology instrument that has been previously validated for use in dogs. The LOAD is a simple descriptive scale completed by owners characterizing joint pain, joint dysfunction, and the impact of OA on daily activity. Any dog within the healthy control group with a LOAD score of >0 was excluded from the study.

3.3.6 Statistical Analysis – Statistical analyses were performed using SAS V 9.3 (Cary, NC). A power analysis was performed utilizing previously published values for canine serum leptin. A sample size of 18 dogs per group was determined to achieve 80% power to detect differences at a significance of $\alpha=0.05$. Student *t*-tests were used to test for differences in SF leptin means due to OA status (healthy versus OA), presence of IL-1β in serum or SF, and the potential confounder of NSAID use ($p<0.05$). Linear regression was used to test for the association of SF leptin with serum leptin, BCS, body fat %, LOAD, and radiographic score (OA group only) and the potential confounder Ω6:Ω3 ($p<0.05$). Multiple linear regression was used to adjust the linear models for the association of SF leptin with the primary variables of interest for confounding factors

(p<0.10). Multivariable linear regression was also used to adjust the model for the association between SF leptin and OA status for BCS (p<0.05).

3.4 Results

Samples were successfully collected from all 48 dogs. Age was not significantly different between the control (4.8 ± 2.97 years) and OA (5.8 ± 2.8 years) groups (p = 0.18). Body condition score was significantly higher in the OA group (6.8 ± 1.4), compared to the control group (5.7±1.0), respectively (p<0.001). Dietary Ω-6 to Ω-3 ratio was determined to be a confounding factor as synovial fluid leptin significantly increased with Ω-6:Ω-3 (r=0.4637; p=0.003). For every 1 unit increase in dietary Ω-6:Ω-3, a 0.5ng/mL increase in SF leptin was found (R^2= 0.207; p=0.0025). Therefore, dietary Ω-6:Ω-3 was included in further multiple linear regression analysis.

SF leptin was significantly lower but positively associated with serum leptin (**Table 3.1** and **Fig. 3.1**). For every 10ng/mL increase in serum leptin there was a 5.8ng/mL increase in SF leptin (R^2=0.581; p<0.0001). SF leptin was positively correlated with measures of body habitus including BCS (R^2=0.366; p=0.0004) (Fig. 2) and body fat % (R^2=0.235; p=0.04). For every 1 unit increase in BCS, a 1.7ng/mL increase in SF was found and for every 10% increase in body fat % SF leptin increased by 2.7ng/mL.

Synovial fluid leptin was significantly higher in dogs with OA by a mean amount of 2.8ng/mL (95% CI 0.6-5.1); however, this difference decreased and was no longer significant when adjusted for difference in BCS between OA and healthy groups (p=0.25). There was a weak positive association between SF leptin and LOAD (R^2=0.091; p=0.03) and LOAD increased by 10 points for every 1ng/mL in SF leptin.

There was no significant correlation between SF leptin and serum or SF IL-1β (p= 0.73, p=0.64, respectively). Additionally, no association was found between SF leptin and radiographic score or NSAID use (p=0.15 p=0.55, respectively).

3.5 Discussion

This is the first study to report the presence of leptin in canine SF. The finding of higher concentrations of SF leptin in obese dogs compared with ideal dogs supported our first hypothesis. Synovial fluid leptin concentrations seemed mostly influenced by serum leptin and body fat percentage because SF leptin paralleled serum leptin concentrations. Plasma leptin strongly correlated with body fat content via hydrometry and BCS in previous canine studies [22]. In addition, higher serum leptin was documented in chronically obese dogs compared to non-obese dogs [23]. Similarly, to these previous findings, serum and SF leptin concentrations were higher in obese dogs than optimal weight dogs in our population. As SF is an ultra-filtrate of plasma [24], SF leptin likely reflects serum leptin concentrations.

The lack of correlation between OA status and SF IL-1β or radiographic score led us to reject our second hypothesis regarding the role of leptin in the canine OA stifle. This finding is quite different from previous reports in humans with OA; in these studies, both serum and SF leptin concentrations were higher in OA compared to healthy patients [15,25]. While leptin was not increased in OA dogs in our study, a few cytokines have been found to be elevated in canine OA SF. The pro-inflammatory cytokine, IL-1β has been shown to be higher in dogs with CCL injury compared to normal dogs [26]. In addition, in man, stimulation of cartilage with leptin resulted in release of IL-1β [22].

This may reflect a species related difference in leptins role in OA progression and synovial inflammation. In addition, other pro-inflammatory cytokines, Il-6 and TNF-α, found in CCL deficient canine SF, may be more important in leptin mediated synovial inflammation than IL-1β [26].

The lack of correlation between radiographic severity and leptin in our study is interesting but not surprising. In man, data regarding adipokines and radiographic severity of OA vary between studies. At least two independent studies have reported a correlation between radiographic severity and SF leptin [27,28]. However, others have found that there is no association between radiographic severity of OA and SF leptin [29,30]. The role of leptin in OA severity remains unclear in man and dogs, however the results of our study provide evidence to suggest that SF leptin may not be an important factor in determining radiographic severity. The lack of correlation between radiographic severity and leptin in dogs is not surprising because there are minimal predictors of radiographic severity and progression in dogs. In man, low levels of C-reactive protein may predict OA progression in early OA [31]. In addition, synovial fluid ARGS-aggrecan fragment (N-terminal sequence of aggrecan containing the amino acids alanine, glycine, and serine), which is formed by cleavage of aggrecan by metalloproteinases and other aggrecanases, has been inversely associated with joint space narrowing but not osteophyte production or overall radiographic severity of OA [32]. Unfortunately, there are limited biomarkers available for prediction of OA severity and progression in both dogs and people.

Synovial fluid leptin correlated weakly with LOAD score despite its lack of association with radiographic severity or synovial inflammation. The relationship

between leptin and owner reported pain and joint dysfunction is currently unclear. Synovial fluid leptin levels, as indicated by other findings reported here, may purely reflect body fat percentage and BCS. In dogs, it has been reported that stifle biomechanics are different in obese compared to ideal weight dogs and obese dogs display great loading forces on the limbs [36]. Therefore, the relationship between LOAD score and SF leptin may represent a biomechanical link between obesity and pain associated with OA. However, the possibility of a proinflammatory effect of leptin within the canine joint cannot be eliminated because there are currently no published studies reporting the effect of leptin on canine cartilage or synovium. In addition, the lameness and perceived joint discomfort may have been due purely to CCL injury and not OA.

Dietary Ω-6:Ω-3 was considered as a potential confounding factor that was evaluated in this study. Indeed, diets high in Ω-3 fatty acids and a low Ω-6 to Ω-3 ratio have led to an improvement in clinical lameness in dogs with OA [34,35]. High concentrations of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) Ω-3 fatty acids, inhibited cartilage release of glycosaminoglycans in response to IL-1β and/or leptin stimulation [36]. In our study, a 0.5-ng/mL decrease in SF leptin was seen with every one-unit decrease in Ω-6:Ω-3. The correlation between dietary fatty acids and SF leptin justified the inclusion of Ω-6:Ω-3 as a confounder in subsequent linear regression models. The low number of dogs consuming a diet high in EPA and DHA in our cohort affected the evaluation of the effects of dietary fatty acids. However, the correlation detected here is interesting and consistent with studies in man that have noted a decrease in serum leptin after supplementation with EPA and DHA [37,38]. Additional studies are warranted to elucidate the effects of Ω-3 and Ω-6 on canine serum and SF adipocytokines.

A major limitation of this study is the subjective nature of the BCS method for evaluating obesity in dogs. Three trained observers were used to attempt to reduce some subjectivity of the evaluation. Several studies have established the strong correlation between objective measures of canine body fat, such as dual-energy x-ray absorptiometry (DEXA) and deuterium (D_2O) dilution, and BCS in dogs [22,39]. In addition, morphometric measures were used and have also shown strong significant correlation to DEXA body condition measurements [20,39]. Another limitation is the absence of radiographs of stifles selected for arthrocentesis in our healthy dogs. Although we cannot ascertain the absence of pathology, OA is not a primary condition in dogs, and we believe that conditions typically leading to OA would have been detected with a thorough lameness evaluation and orthopedic examination. Finally, the relationship between SF leptin and objective measures of gait (i.e. kinetic gait analysis) were not evaluated in this study. However, LOAD is considered a well-validated clinical metrology instrument in dogs [40].

In this study, we determined that SF leptin was well correlated serum leptin and measures of body habitus in both healthy and secondary osteoarthritic canine joints. In addition, a positive association was found between LOAD scores and SF leptin in osteoarthritic joints. While further studies are necessary to determine whether this correlation is due to purely a biomechanical change related to obesity or whether leptin has a proinflammatory role in canine joints, this study highlights the potential significance of SF leptin on owner-perceived joint pain and dysfunction and highlights the requirement of a strict weight loss regimen in the clinical management of canine OA.

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Table 3.1. Body condition score and concentrations of cytokines in healthy dogs, dogs with OA, and in both groups combined. Values with superscript a are significantly different ($p<0.0001$). Values with superscript b were significantly different; however, this difference was no longer significant when adjusted for difference in BCS among groups ($p=0.25$). Values with superscript c are significantly different ($p<0.05$)

	Control		OA		All	
	Mean	SD	Mean	SD	Mean	SD
Synovial Fluid Leptin (ng/mL)	2.4[b]	2.04	5.23[b]	4.6	4.09[a]	4
Serum Leptin (ng/mL)	4.9	4.3	8.13	5.9	6.88[a]	5.52
Body Condition Score	5.72[c]	1.00	6.76[c]	1.40	6.33	1.36
Synovial Fluid IL-1 (pg/mL)	5.73	18.11	82.35	284.66	62.19	245.72
Serum IL-1 (pg/mL)	16.44	48.59	215.42	681.07	140.80	544.56

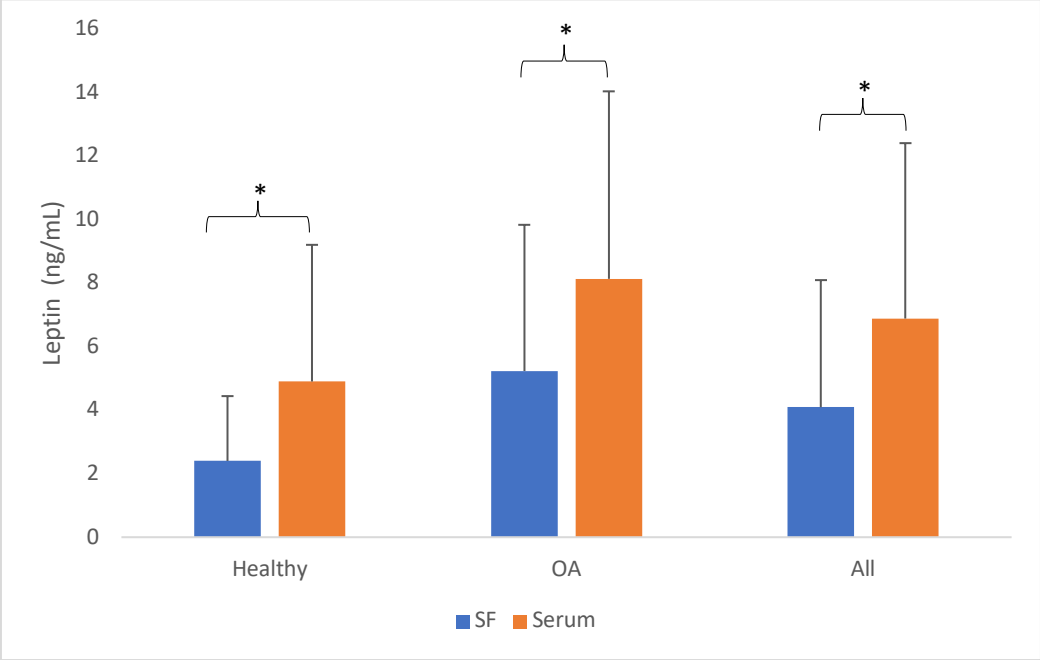

Figure 3.1 Serum and Synovial Fluid Leptin Concentration (Mean +/- SD) in Healthy Dogs, Dogs with OA, and in Both Groups Combined. Student's t test was done for statistical analysis. * indicates statistical difference between serum and SF leptin, $p<0.05$. SF = synovial fluid

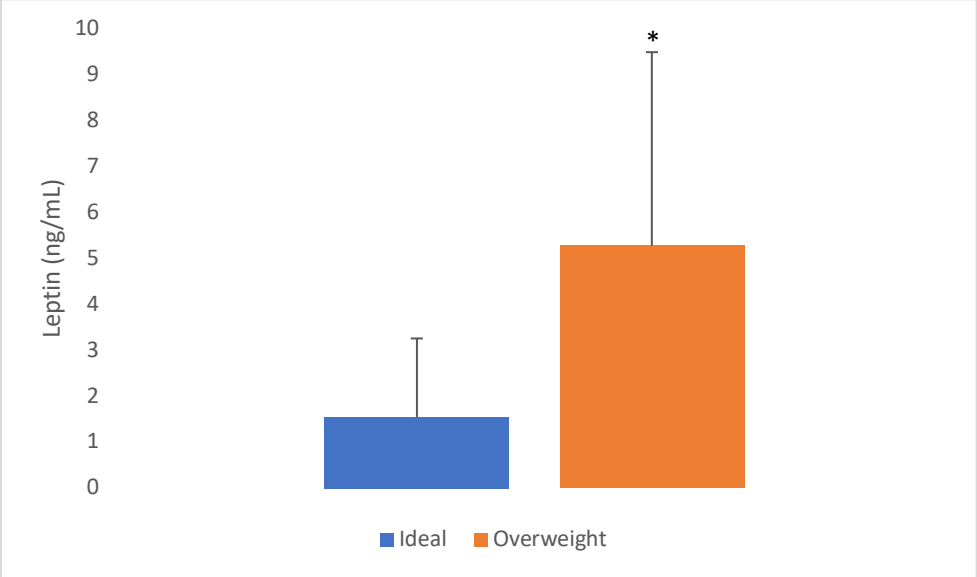

Figure 3.2 Synovial fluid leptin concentrations based on body condition score groups in healthy dogs and those with OA. Data is presented as mean ± SD. Student's t was performed to test for difference amongst groups. *Indicates statistical difference between groups, p=0.004.

CHAPTER 4

ELEVATED SYNOVIAL FLUID CONCENTRATION OF MONOCYTE CHEMOATTRACTANT PROTEIN-1 AND INTERLEUKIN-8 IN DOGS WITH OSTEOARTHRITIS OF THE STIFLE

Kleine, S.A., Gogal Jr., R.M., George, C., Thaliath, M.A., Budsberg, S.C. Submitted to Veterinary Comparative Orthopaedics and Traumatology

4.1 Abstract

Chemokines such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) have been shown to cause monocyte and NK cell chemotaxis and polymorphonuclear cell chemotaxis, respectively. Additionally, MCP-1 signaling has been implicated in modulating pain. Elevated synovial fluid concentrations of MCP-1 and IL-8 have been demonstrated in humans with osteoarthritis (OA), but currently there are no studies evaluating synovial MCP-1 or IL-8 concentrations in dogs. Additionally, there are no canine studies evaluating the correlation between these chemokines and caregiver perceived pain and mobility, as measured by the clinical metrology instrument, Liverpool Osteoarthritis in Dogs (LOAD). This study documented elevated synovial fluid concentrations of IL-8 and MCP-1 in the stifle of dogs with secondary OA compared to normal stifles. However, this study found no correlation between MCP-1 or IL-8 and LOAD or radiographic severity of OA.

4.2 Introduction

Osteoarthritis (OA) is a common cause of pain, decreased mobility, and joint dysfunction in the canine population. Despite its prevalence and significant health implications, the pathophysiology is still not fully elucidated. Additionally, the inflammatory cascade involved in canine OA has not been fully evaluated and no inflammatory mediators have been found to correlate with the radiographic severity of OA in dogs. Characterization of inflammatory cytokines and chemokines involved in the pathogenesis of OA may lead to more targeted therapeutic interventions.

Inflammatory mediators, such as TNF-α and IL-6, have been evaluated in canine OA synovial fluid. Two separate studies have reported elevated IL-6, in synovial fluid from dogs with experimentally-induced and naturally occurring OA compared to synovial fluid from healthy dogs [1,2]. IL-6 was found to be directly proportional to Glycosaminoglycan synthesis in the experimental OA group [2]. Both of these studies also quantified synovial fluid TNF-α; however one study reported elevated concentrations of TNF-α in the OA group[2], while the other reported lower concentrations in the OA group compared to the control dogs [1].

Chemokines in synovial fluid have also received some attention in the scientific literature. Monocyte chemoattractant protein-1, MCP-1 (CCL2), is a chemokine that induces monocyte and NK cell chemotaxis [3], while IL-8 (CXCL8) is chemotactic for polymorphonuclear cells [4]. In humans, MCP-1 has been found in synovial fluid from OA patients and is elevated compared to control patients [5]. Additionally, MCP-1 signaling modulates pain and blockade of MCP-1 attenuates cartilage mononuclear cell infiltration and destruction [5]. Higher concentrations of IL-8 have been reported in human OA synovial fluid compared to healthy patients [4]. Results from one study evaluating chemokine and cytokine RNA in canine OA synovial fluid found increased expression of IL-8 [6]. Presently, there are no studies evaluating MCP-1 or IL-8 concentration in synovial fluid from OA compared to healthy canines.

In the present study, concentrations of TNF-α, MCP-1, granulocyte monocyte - colony stimulating factor, KC-like, IP-10, and interleukins 2, 7, 8, 10, 15, and 18 were quantified in the serum and synovial fluid of normal dogs and dogs with secondary stifle

OA. Of particular interest, were the TNF-α, MCP-1, IL-6, and IL-8 concentrations. We hypothesized that TNF-α, IL-6, IL-8, and MCP-1 synovial fluid, but not serum concentrations would be elevated in dogs with secondary OA as compared to synovial fluid from normal stifles.

4.3 Materials and Methods

4.3.1 Animals

Thirty-nine dogs of various breed, age, and sex (13 healthy controls and 26 with CrCL injury and secondary OA) were enrolled in the study. For all dogs, a general physical and orthopaedic examination together with routine haematology was performed. Any dog with evidence of systemic disease was excluded from the study. Dogs owned by staff, student, and faculty members at the University of Georgia were recruited for inclusion in the healthy group. They were excluded if there was any evidence of lameness or orthopaedic disease. The OA dogs were client-owned dogs presenting for unilateral cranial cruciate ligament insufficiency and secondary OA. Dogs in the OA group were excluded if there was evidence of any orthopaedic disease other than unilateral CrCL insufficiency and secondary OA. The study was reviewed and approved by the University of Georgia, Clinical Research Committee. All owners signed informed consent documents after complete explanation of the protocol.

4.3.2 Sample collection

Healthy dogs were lightly sedated with dexmedetomidine (5mcg kg^{-1} I.V.). A single stifle was arbitrarily selected and was clipped and aseptically prepared. A standard stifle arthrocentesis was performed. The synovial fluid was aspirated and immediately placed on ice. For dogs in the OA group, synovial fluid was collected aseptically from the affected stifle intra-operatively prior to arthrotomy. Joint lavage was not performed in any dog. Ten milliliters of blood was collected from all dogs via standard venipuncture and placed into tubes containing no additives.

Sample processing and storage

To separate cellular contaminants from the synovial fluid, samples were centrifuged at 4°C at 4000xg for 10 minutes. Only clear and yellowish supernatants were selected for further analysis. Samples were subdivided into 200uL aliquots, to minimize freeze-thaw cycles. Samples were stored at -80°C until analysis.

4.3.3 Radiographic evaluation

Standard orthogonal, mediolateral and craniocaudal radiographs were obtained from the affected stifle of dogs in the OA group. The radiographs were evaluated by a radiologist (CG) using a previously established scoring system (Appendix 1) [6].

4.3.4 Client questionnaires

All caregivers were required to complete a questionnaire regarding diet history (Appendix 2). This allowed for determination of dietary Ω-3 and Ω-6 on a dry matter

basis, utilizing published manufacturer data. When no published Ω-3 and Ω-6 values were readily available, the food manufacturer was contacted directly.

In the OA group, caregivers were additionally required to complete a previously validated clinical metrology instrument, Liverpool Osteoarthritis in Dogs (LOAD).

4.3.5 Cytokine assay

Synovial fluid and serum were analyzed using a 13-plex canine cytokine/chemokine MILLIPLEX™ MAP Kit assay (Millipore Sigma, Darmstadt, Germany). According to the manufacturer, the human MILLIPLEX™ MAP assay utilizing the same test procedure and reagents with the exception of the species-specific antibodies, has been previously validated for use in synovial fluid [7]. The cytokines and chemokines evaluated included IL-2, IL-6, Il-7, IL-8, IL-10, IL-15, IL-18, IFNγ, TNF-α, GM-CSF, KC-like, IP-10, and MCP-1. Both serum and synovial fluid was diluted 1:3 and the assay was performed according to manufacturer's instructions. The concentration of each analyte was calculated using a standard curve generated from provided standards and a blank. For any sample that was out of the detectable range, the highest or lowest extrapolated value was assigned to that sample. Due to cost constraints, samples were not retested at a different dilution. The values were reported as pg/mL.

4.3.6 Statistics

Comparison of synovial fluid and serum cytokines and chemokines from the two groups was performed using Mann-Whitney tests. Spearman's rank-order correlation was used to

evaluate the relationship between MCP-1, IL-8, and IL-6 and LOAD, radiographic score, and dietary Ω-6:Ω-3. All hypothesis tested were two-sided with significance at α=0.05.

4.4 Results

There was no significant difference in age or sex between the healthy (4.8 ± 2.97 years) and OA (5.8 ± 2.8 years) groups (p=0.18; p=0.22, respectively). Mixed breed dogs were the most prevalent dog breed in both groups (n=6, healthy; n= 13, OA group) followed by Labrador Retrievers (n=5, healthy; n=3, OA). Median (interquartile range [range]) synovial fluid MCP-1 was 0.0 (0.0 [0.0-221.2]) pg/mL for the healthy group and 224.4 (203.2[0-1620.0]) pg/mL in the OA group (**Fig. 1**). Synovial fluid MCP-1 was significantly elevated in the OA group compared to synovial fluid MCP-1 in the healthy group (p<0.0001). Median (interquartile range [range]) synovial fluid IL-8 was 63.2 (114.30 [63.9-4094.0]) pg/mL for healthy dogs and 468.0 (471.9 [63.2-8970.0] in the OA group (**Fig. 2**). Synovial fluid IL-8 was significantly lower in the healthy group compared to the OA group (p=0.005). Median (interquartile range [range]) synovial fluid IL-6 was 0.0 (0.0 [0.0]) pg/mL for healthy dogs and 15.2 (75.9 [0.0-393.3] in the OA group. Synovial fluid IL-6 was significantly lower in the healthy group compared to the OA group (p=0.0005). TNF-α, GM-CSF, IL-2, and IP-10 were below the limits of detection in all synovial fluid samples from both groups. There was no significant difference in synovial fluid IL-7 (p=0.12), IL-10 (p=0.06), IL-15 (p=0.24), IL-18(p=0.17), KC-like (p=0.06), or IFNγ (p=0.36) based on OA status. Spearman's rank-order correlation found no correlation between synovial fluid MCP-1, IL-8, or IL-6 and LOAD or radiographic

score in the OA group and dietary Ω-6:Ω-3 in all dogs. There was no significant difference in any serum cytokine or chemokine concentration in dogs with OA compared to healthy dogs. Additionally, there was no correlation between serum and synovial fluid IL-6 (p=0.33), IL-8 (p=0.06), or MCP-1 (p=0.05).

4.5 Discussion

This data supports our hypothesis that increased synovial fluid IL-8, MCP-1, and IL-6 concentrations are present in dogs with OA compared to normal dogs. However, we rejected our hypothesis that TNF-α would follow the same trend, as TNF-α was not detectable in any synovial fluid sample. Additionally, these data demonstrate a lack of correlation between synovial fluid IL-8, MCP-1, or IL-6 and radiographic OA severity or joint pain and dysfunction, as measured by LOAD.

The evaluation of cytokine and chemokine changes within synovial fluid may help to elucidate the underlying mechanisms for the pathophysiologic changes seen in canine OA. The results of this study are similar to findings in human OA, where MCP-1 and IL-8 have been found to be elevated in OA synovial fluid [8,9]. However, the lack of correlation between either IL-8 or MCP-1 and LOAD are in contrast to studies in human OA [8,9], where both chemokines were associated with pain. In this study, an objective measure of lameness (i.e. kinetic gait analysis) was not used to determine the correlation between pain and synovial fluid MCP-1, IL-8, or IL-6. Despite previous validation of the LOAD, objective gait analysis may have allowed for detection of small differences in lameness.

While this study highlights 3 cytokines that are elevated in canine OA, the role of these cytokines in joint biochemical changes was not explored. As both MCP-1 and IL-8 have been implicated in fibroproliferative changes in canine idiopathic pulmonary fibrosis [10] future studies evaluating their role in fibroproliferative changes in canine OA are warranted. Despite IL-8s classic role in neutrophil chemotaxis, neutrophil populations are consistently low in OA synovial fluid and the synovial membrane; however, IL-8 has been implicated in increased type X collagen expression, nitric oxide production, and expression of MMP-13 and tissue inhibitor of metalloproteinase-3 in both human and bovine articular chondrocytes [11]. This suggests a possible regulatory role of IL-8 within the joint. Additionally, in contrast to previous studies [1,2], TNF-α was not detectable in the synovial fluid from any dog in this population. Only one study has a reported a difference in synovial fluid TNF-α in experimental canine OA compared to healthy controls. In that study, TNF-α was measured 3 months post CrCL transection [2]. In our study the dogs with naturally-occurring CrCL injury, presented with varying durations of injury chronicity with a majority suffering from CrCL rupture exceeding 3 months duration. This may account for the differences in synovial fluid TNF-α amongst the two studies.

A limitation of this study was that dogs in the OA group were not breed matched. Breed specific differences in inflammatory mediators may have played a role in the differences in this study; however, given that a majority of dogs in both groups were mixed breeds the effect of breed differences is likely minimal. A second limitation of the study was that cell counts were not performed on the synovial fluid to eliminate the possibility of cellular release of chemokines via cell contaminants. Still an aggressive

was made to remove any cellular contaminants in the synovial fluid. Each sample was immediately centrifuged and the supernatant was carefully collected and used for further analysis, as previously described [6]. Given the lack of correlation between serum and synovial fluid cytokines / chemokines in this study, blood contamination resulting in elevated synovial fluid concentrations is likely negligible.

In this study, we have provided initial evidence that IL-8 and MCP-1 are elevated in canine OA synovial fluid. However, continued work is needed to determine the role of MCP-1 and IL-8 on OA-associated pain and synovial proliferation.

4.6 References

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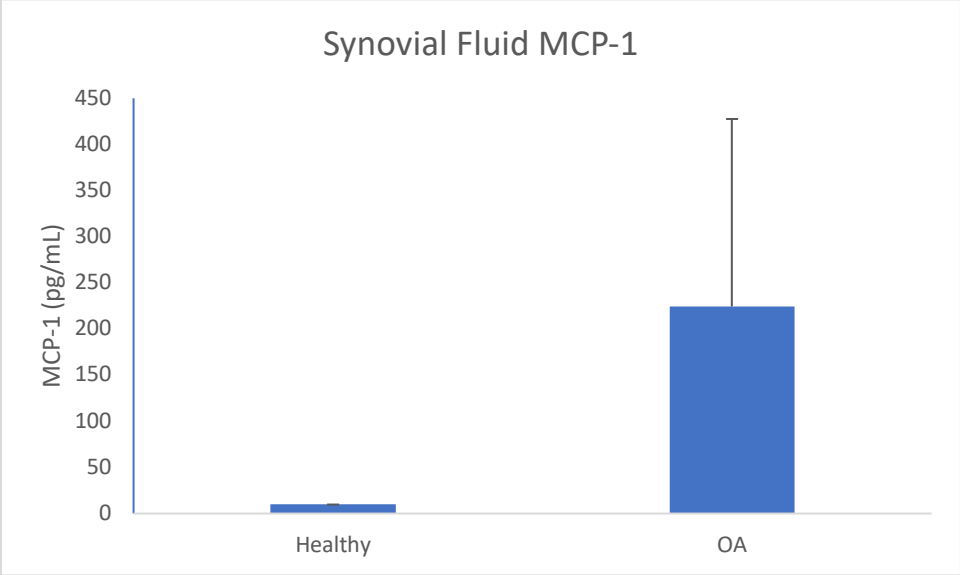

Figure 4.1 Stifle joint synovial fluid monocyte chemoattractant protein-1 (MCP-1) concentrations from two groups of dogs (healthy and osteoarthritis). Data is presented as median and interquartile range. Mann Whitney U test was performed for statistical analysis; $p<0.0001$.

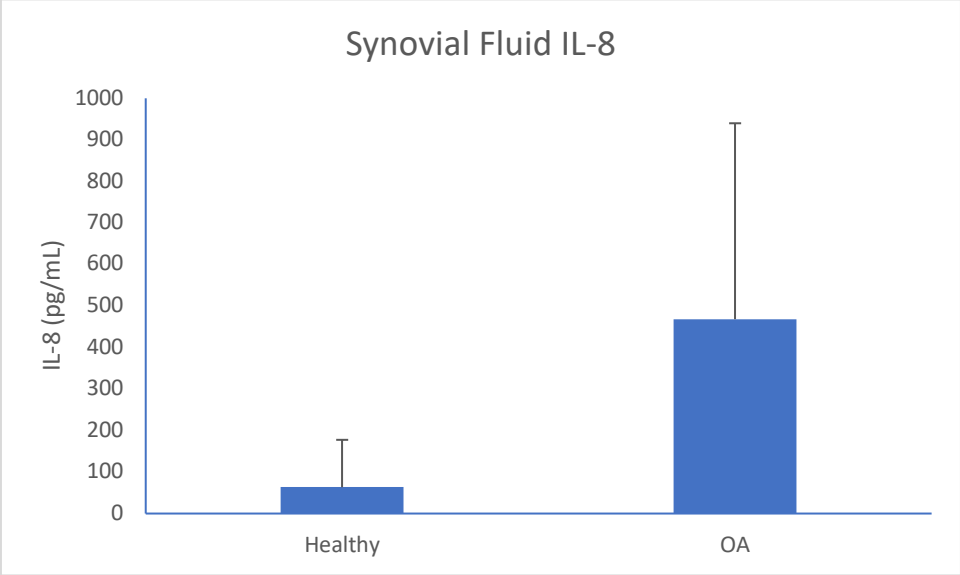

Figure 4.2 Stifle joint synovial fluid IL-8 concentrations from two groups of dogs (healthy and osteoarthritis). Data is presented as median and interquartile range. Mann Whitney U test was performed for statistical analysis; p=0.005

CHAPTER 5

RELATIONSHIP BETWEEN SERUM AND SYNOVIAL FLUID RESISTIN CONCENTRATIONS AND BODY CONDITION SCORE AND OSTEOARTHRITIS STATUS IN DOGS

Kleine, S.A., Gogal Jr., R.M., Krunkosky, T.M., Sanderson, S.L., George, C., Norton, M.M., Budsberg, S.C. Submitted to Journal of Veterinary Internal Medicine

5.1 Abstract

Background: While resistin is classified as an adipokine, in humans it functions as a proinflammatory mediator released from monocytes and macrophages. Conflicting evidence exists regarding resistin's link with adiposity in humans and canines. Human synovial fluid (SF) resistin is associated with osteoarthritis (OA) severity and joint inflammation. Presently, little evidence exists regarding canine resistin.

Objectives: Evaluate resistin in healthy versus OA canine SF. To determine the correlation of serum resistin with body condition score (BCS). To assess resistin release from peripheral blood mononuclear cells (PBMCs) and adipocytes.

Animals: Thirteen healthy and 26 OA client-owned dogs were enrolled. Blood was collected from 6 healthy purpose-bred dogs for PBMC culture. Six mixed-breed dogs were used for adipose tissue collection and adipocyte culture.

Methods: A prospective, controlled clinical trial was used to measure resistin in canine serum and SF. Resistin concentrations were measured in healthy and OA SF. Serum and SF resistin were evaluated according to BCS. PBMCs and adipocytes were cultured under 3 conditions, media alone, media + lipopolysaccharide, and media + Concanavalin A (Con A). Resistin concentrations were measured with a canine specific enzyme-linked immunosorbent assay.

Results: Resistin concentrations in OA SF were comparable to healthy SF. Neither serum nor SF resistin concentrations correlated with BCS. Cultured enriched PBMCs stimulated with Con A released resistin, while adipocytes did not.

Conclusions and Clinical Importance: Resistin does not appear to have a significant role in OA severity in the dog nor does is it correlate with BCS. A novel finding is that resistin is released from stimulated T cells but not stimulated adipocytes.

5.2 Introduction

Resistin is a cysteine-rich molecule that is expressed in high levels in mouse white adipose tissue (WAT). Due to the high expression of resistin within rodent WAT, this molecule has been classified as an adipocytokine. In addition, resistin concentrations paralleled obesity in this population of mice, further supporting its role as an adipocytokine [1]. Despite these findings, the role of resistin has not been clearly elucidated, but it is thought to be involved in insulin resistance [1-3].

In humans, studies regarding resistin and the correlation with adiposity are conflicting, with some studies reporting a lack of correlation with measures of adiposity [3,4] and some reporting a correlation [5]. Interestingly, one study reported a negative correlation between body mass index and serum resistin [6]. The *in vitro* discovery that resistin in humans is actually released from monocyte / macrophage cell lines may explain this discrepancy [7]. In support of this, another study showed that WAT did not express resistin mRNA [8]. Additionally, with obesity, the influx of macrophages into WAT correlated increase in resistin with increasing adiposity, as greater numbers of

activated macrophages are available to release this molecule [9]. Similar to several human studies, one study in dogs found a lack of correlation of serum resistin with body condition score (BCS) [10].

While most rodent and select human studies report an association between resistin and insulin resistance [1,3], resistin is recognized a major pro-inflammatory adipocytokine that results in release of TNF-α , IL-6 and IL-1β [11]. As osteoarthritis (OA) has an inflammatory component to the pathophysiology, the link between OA and this cytokine has been evaluated only in certain species. In humans, there is an association between both serum and synovial fluid (SF) resistin and the development of OA. Serum resistin has been shown to be a risk factor for the development of hand OA and has been associated with cartilage damage [12]. Synovial fluid resistin has been linked to clinical severity of OA [13,14], cartilage damage and SF IL-6, MMP-1 and MMP-3 [15].

In canines, there is a series of studies that demonstrated that dogs with a higher caloric intake had a higher incidence of shoulder and coxofemoral OA, which was more severe in the coxofemoral joint of overweight dogs compared to their lean cohorts [16,17]. Additionally, obese dogs have been shown to have greater range of motion at the hip, hock, and shoulder than lean dogs [18]. While there is some evidence that obesity is important in canine OA, there is no evidence regarding the role of adipocytokines in the development or progression of this disease.

The primary goal of this study was to quantify serum and SF resistin and evaluate whether there is a correlation between resistin and OA status in dogs. We hypothesized

that resistin concentrations would be higher in dogs with OA compared to healthy dogs. Given the lack of correlation with BCS in a previous study [10], our second goal was to determine whether resistin was different based on BCS and body fat percentage in dogs. We hypothesized that serum and synovial fluid resistin would not be different based on BCS and show no correlation with body fat percentage. Our last goal was to assess whether resistin is released from canine peripheral mononuclear cells and canine WAT derived adipocytes. Our last hypothesis was that monocytes would be the main source of resistin release within the dog.

5.3 Materials and Methods

5.3.1 Animals – Thirty–nine client owned dogs, 13 healthy controls (non-OA) and 26 with cranial cruciate ligament (CrCL) injury and secondary OA (OA group), were enrolled for the first portion of the study. The non-OA group was determined to be healthy on the basis of a physical exam, routine hematology, and a complete orthopedic exam. Any dog with a history of lameness or pain on palpation of the joint was excluded from enrollment. For the OA group, dogs presenting for surgical correction of CrCL injury, were enrolled. Dogs were deemed to be otherwise healthy based on physical exam and routine hematology. Dogs in the OA group were excluded if there was evidence of any systemic disease or orthopedic disease of any joints other than stifle. Additionally, dogs weighing less than 8kg in either group were excluded due to the inability to collect adequate volumes of synovial fluid. Signed informed owner consent was obtained prior to enrollment. For the peripheral blood mononuclear cell (PBMC) culture portion of the

study, 6 purpose-bred healthy hound mixes, weighing between 21-39kg, were enrolled. For the adipocyte cell culture, 6 healthy skeletally-mature mixed breed dogs were enrolled. All procedures were approved by the University of Georgia Animal Care and Use Committee.

5.3.2 Sample Collection – Dogs in the non-OA group were sedated with dexmedetomidine (5mcg kg^{-1} IV). Blood was collected via standard jugular venipuncture. A single femorotibial joint was arbitrarily selected for arthrocentesis. The skin over the joint was shaved and aseptically prepared. Synovial fluid was collected via routine arthrocentesis and joint lavage was not performed for fluid collection in any dog. For dogs in the OA group, blood collection via standard jugular venipuncture was performed just after anesthetic induction. Synovial fluid was obtained intra-operatively just prior to arthrotomy. Dogs in both groups were fasted for 8 hours prior to sample collection. All synovial fluid and blood samples were placed on ice immediately following collection. Synovial fluid samples were centrifuged at 4°C at 4000 x g to separate cells and debris. Only samples that were clear to yellowish in color were stored for later processing. All samples were divided into 250µL aliquots to minimize freeze-thaw cycles, then stored at -80°C until analysis.

5.3.3 Radiographic Evaluation – Standard orthogonal, mediolateral and craniocaudal, radiographs were performed of the affected stifle of dogs within the clinical OA group. The radiographs were evaluated by a single radiologist utilizing a previously established scoring system (Appendix 1) [19].

5.3.4 Client Questionnaires – All caregivers were required to complete 2 forms, a diet history questionnaire (Appendix 2) and the Liverpool Osteoarthritis in Dogs (LOAD). The diet questionnaire allowed for determination of dietary Ω-3 and Ω-6 on a dry matter basis, utilizing published manufacturer data. If there was no published data regarding Ω-3 and Ω-6 was readily available, the food manufacturer was contacted directly. Caregivers of dogs within the OA group also completed the LOAD. This a previously validated clinical metrology instrument [20].

5.3.5 Peripheral Blood Mononuclear Cell Culture – Twenty milliliters of blood were collected aseptically via routine jugular venipuncture. Blood was placed into tubes containing potassium EDTA. The blood was centrifuged at 283 x g for 30 minutes at 23°C. The plasma fraction was removed and the cellular fraction was reconstituted 1:3 with phosphate buffered saline. This was then slowly layered over a density gradient medium (Histopaque-1077) and centrifuged at 23°C at 450 x g for 30 minutes. The buffy coat was collected and cells were washed twice. For washing, the cells were resuspended in 7mL cold Dulbecco Modified Eagle Medium (DMEM) and centrifuged at 8°C at 395 x g for 15 minutes. The cells were resuspended in complete culture medium consisting of

DMEM with 10% FBS, 2% penicillin-streptomycin and 1% nonessential amino acids. The cells were enumerated (Nexcelom electronic cell counter, Nexcelom Bioscience, Lawrence, MA, USA) and cultured at a density of 1.25 x 10^6 cells per well. PBMC from each dog were cultured with 5 separate treatment conditions: media alone (negative control), Concanavalin A (Con A) 1μg/mL, lipopolysaccharide (LPS) 1000ng/mL, leptin 20ng/mL, and leptin 40ng/mL for 24 hr. Supernatants were collected and stored at -80°C until analysis.

5.3.6 Adipocyte Culture – Canine adipocytes were cultured from 6 healthy mixed-breed dogs utilizing a previously published protocol [21,22]. Two to five grams of white adipose tissue was sterilely dissected from peri-uterine or peri-testicular tissue at the time of routine sterilization. Samples were placed in phosphate buffered saline (PBS) and placed on ice for transport to the lab. Each tissue was washed 3 times with PBS and debrided of connective tissue and blood vessels. The tissue was minced into small pieces and incubated in 15mL of 1mg/mL collagenase for 60 minutes at 37°C on a shaking platform at 60 shakes / minute. After collagenase incubation, samples were centrifuged at 200 x g at 23°C for 10 minutes. The supernatant was aspirated and discarded. The remaining pellet was suspended in 5mL inoculation medium consisting of basal medium (DMEM, 15mM HEPES, 15mM $NaHCO_3$, 33μM biotin, 17μM D-pantothenate and 2.5μg/mL amphotericin) plus 10% fetal bovine serum (FBS), 100U/mL penicillin, and 100μg/mL streptomycin. Each resuspended sample was filtered through a 100μm nylon screen and treated with erythrocyte lysis buffer for 10 minutes at 23°C. Each cell

suspension was then washed twice with PBS and resuspended in 3mL of basal medium. The cells were enumerated (Nexcelom electronic cell counter, Nexcelom Bioscience, Lawrence, MA, USA) and suspended in inoculation medium in order to achieve a plating density of 40,000-60,000 cells/cm^2. The cells were incubated for 24hr at 37°C with 5% CO_2 and 95% air. After 24hr, the cells were again washed 3 times with PBS and a single sample from each dog was fixed with 3.7% formaldehyde at room temperature for > 1 hour. Each single well of fixed cells was washed twice with PBS and stained with Oil Red O for 1hr at 23°C and then washed twice with PBS. After staining, the cells were examined under light microscopy and images of adipocytes were obtained (**Fig. 1**). In the remaining wells, inoculation medium was replaced with induction medium, consisting of basal medium with 2% FBS, 10µg/mL insulin, 1nM 3,3', 5-triido-L-thryonine (T3), 0.5mM IBMX, 1µg/mL rosiglitazone, 100U/mL penicillin, 100µg/ml streptomycin, and 2.5µg/mL amphotericin (Day 0). On day 3 the induction medium was replaced with incubation medium, which consisted of basal medium with 2%FBS in basal medium, 10µg/mL insulin, 1nM T3, 100U/mL penicillin, and 100µg/mL streptomycin. Media was replaced with incubation medium every 2 days and the cells were evaluated under light microscopy to ensure adequate adipocyte cell numbers were still present. On day 12, the cells were pre-incubated with FBS-deficient medium for 24hr. On day 13, FBS-deficient medium was removed and replaced with 500µL incubation medium with one of three treatment conditions, media alone (negative control), Con A 1µg/mL, LPS 1000ng/mL. Adipocytes from each dog were treated with each treatment condition in duplicate. The supernatants were removed after 24hr of treatment (day 14) and stored at -80°C until analysis.

5.3.7 Assays – Serum, synovial fluid, and PBMC and adipocyte cell culture supernatant resistin concentrations were determined via a canine specific ELISA (Millipore Sigma, St Louis, MO, USA). Optical density was measured utilizing a plate reader (Biotek Synergy 4, BioTek Instruments, Inc, Winooski, VT, USA) and associated software (Gen 5, BioTek Instruments, Inc, Winooski, VT, USA). A 13-plex canine cytokine / chemokine MILLILEX^{TM} MAP kit assay (Millipore Sigma, St Louis, MO, USA) was used to evaluate synovial fluid samples. The cytokines analyzed included IL-2, IL-6, IL-7, Il-8, IL-10, IL-15, IL-18, IFNγ, TNF-α, GM-CSF, KC-like, IP-10, and MCP-1. All samples were diluted 1:3 and the assay was performed according to manufacturer's instructions. The concentration of all analytes was calculated using a standard curve generated from provided standards.

5.3.8 Statistical Analysis – Student *t*-tests were used to test for differences in resistin means due to OA status (healthy versus OA), BCS, or the potential confounder of non-steroidal anti-inflammatory use. Spearman's correlation was used to determine the correlation between SF resistin and LOAD, radiographic score, body fat percent, and dietary Ω-6:Ω-3. A linear mixed model was used to compare PBMC resistin concentration between treatments and multiple comparisons were adjusted for using Tukey's test. Adipocyte cell culture resistin concentration between treatments was analyzed with a repeated measures ANOVA. All hypothesis tested were two-sided with significance at α=0.05.

5.4 Results

5.4.1 Resistin and OA status

There was no significant difference in age or sex between the healthy (4.8 ± 2.97 years) and OA (5.8 ± 2.8 years) groups (p=0.18; p=0.22, respectively). Mean (SD) synovial fluid resistin was 6472.37 (± 1345.08) pg mL^{-1} in healthy group and 5567.95 (± 3444.71) pg mL^{-1} in the OA group (**Fig. 2**). There was no significant difference in synovial fluid resistin between the healthy and OA group (p=0.08). Within the OA group synovial fluid resistin was not different based on non-steroidal anti-inflammatory use (p=0.99) and therefore was not considered a confounding factor in further analysis. There was no correlation between synovial fluid resistin and LOAD (p=0.14), radiographic score (p=0.86), or dietary Ω-6:Ω-3 (p=0.31). Lastly, there was no correlation between synovial fluid resistin and any of the 13 synovial fluid cytokines or chemokines tested.

5.4.2 Resistin and body condition score

Mean serum resistin concentration was 7545 (± 5149) pg mL^{-1}. Mean serum resistin concentration in the ideal weight group was 9656.40 (± 6948.49) pg mL^{-1} and 6874.75 (± 4476.26) pg mL^{-1} in the overweight group. Mean synovial fluid resistin concentration in the ideal weight group was 6773.97 (± 3668.88) pg mL^{-1} and 5515.92 (± 2606 16) pg mL^{-1} in the overweight group. There was no significant difference in serum or synovial fluid resistin concentrations between the ideal weight group and the overweight group (p=0.11,

p=0.23, respectively) (**Fig. 3**). There was no correlation between synovial fluid or serum resistin and body fat percentage (p=0.98, p=0.68, respectively).

5.4.3 Peripheral blood mononuclear cell and adipocyte culture

After 24hr of PBMC culture, treatment with Con A resulted in a significant increase in resistin concentrations compared to media alone (p=0.047) (**Fig. 4**). Treatment with LPS did not result in a significant increase in resistin concentration compared to media (p=0.93). There was no significant difference in supernatant resistin concentration between LPS and Con A treatment groups (p=0.20). Neither Con A nor LPS resulted in significant release from cultured canine adipocytes compared to media alone (p=0.42) (**Fig. 5**).

5.5 Discussion

This is the first study to report the presence of resistin within canine synovial fluid. The results of our study led to rejection of our first hypothesis. There was no significant difference in synovial fluid resistin in dogs with CrCL rupture and secondary OA when compared to healthy controls. This is in contrast to studies in human OA, where synovial fluid resistin has been linked to pain, joint dysfunction [13], and incident radiographic knee OA, independent of BMI [23]. Additionally, synovial fluid resistin in humans has been correlated with matrix metalloproteinase expression, which are important in cartilage breakdown [15], and associated with CTX-II, a biomarker of

cartilage degradation [13]. Given these findings, adipokines likely have an important role in human OA. However, the true role of resistin systemically and within the canine joint is currently unknown and warrant further investigation. Further, these results would suggest that the dog may not be an ideal model for human OA pathogenesis.

The findings in our study are consistent with earlier evidence in dogs that resistin is not different based on degree of adipocity. In a previous study, BCS did not correlate with resistin in healthy dogs [10]. In our study, resistin was not different based on weight class (ideal versus overweight) and showed no association with body fat percentage. These results along with reports from human literature led to the question of whether resistin is truly an adipokine in the dog. To investigate this question, canine PBMCs were stimulated in culture to quantify resistin release. Unexpectedly, Con A, a T cell mitogen [24], resulted in an increased concentration of resistin, indicating that resistin is released from canine T cells. Interesting, LPS, which primarily binds to TLR4 on macrophage / monocyte cell lines [25], did not result in significant resistin release, pointing to a lack of resistin release from macrophages and monocytes. Despite the fact that this is inconsistent with human data showing resistin release from macrophages and monocytes [7], the release of resistin from canine T cells explains the lack of correlation between resistin and adiposity in dogs. Additionally, stimulated canine adipocytes in culture were unable to increase resistin release, further supporting the implication that resistin is not an adipocytokine in the dog.

In one canine study, serum resistin was determined to be significantly increased in pancreatitis compared with healthy control dogs [26]. Interestingly, histopathologic pancreatic lesions frequently (52.5%) involve lymphocytic infiltration [27]. Given that

our study showed that T cells can release resistin, an increase in the release of resistin in pancreatitis could be potentially be explained by the increases in lymphocytes in this organ. As pancreatitis can represent either an acute or chronic inflammatory condition and canine T cells are capable of resistin release, the role of resistin in inflammation warrants further investigation.

A limitation to this study was the subjective nature of body condition scoring method for evaluating adipocity in dogs. Three individual trained observers all assigned a BCS to each individual dog and morphometric measures were employed, in order to limit some of the subjectivity of this testing method. Additionally, several studies have demonstrated strong significant correlation between objective measures of canine body fat, such as dual-energy x-ray absorptiometry (DEXA) and deuterium (D_2O) dilution, and BCS in dogs [28,29]. Still, the results from our study are consistent with previous work regarding BCS and serum resistin concentrations [10]. Another limitation of this study was the use of Con A and LPS to stimulate resistin release from adipocytes. It is currently unknown what stimulus is required for resistin release from adipocytes; however, a previous study in canine adipocyte culture showed increase expression of leptin from adipocytes after stimulation with LPS [22]. Based on the results of the previous study and our PBMC culture data, Con A and LPS were deemed the most appropriate for eliciting resistin release from adipocytes.

In summary, we determined that synovial fluid resistin concentrations did not correlate with either OA status or BCS in both healthy dogs and those with CrCL rupture and secondary OA. Additionally, there was no association between synovial fluid resistin and LOAD and radiographic score. Since serum and synovial fluid resistin did not

correlate with BCS in this population of dogs, we put forth the possibility that resistin may not be an adipokine in the dog. We did determine that resistin is released from circulating T cells, but not from adipocytes following stimulation with LPS or Con A. Further studies are necessary to determine the role of resistin in obesity and inflammation.

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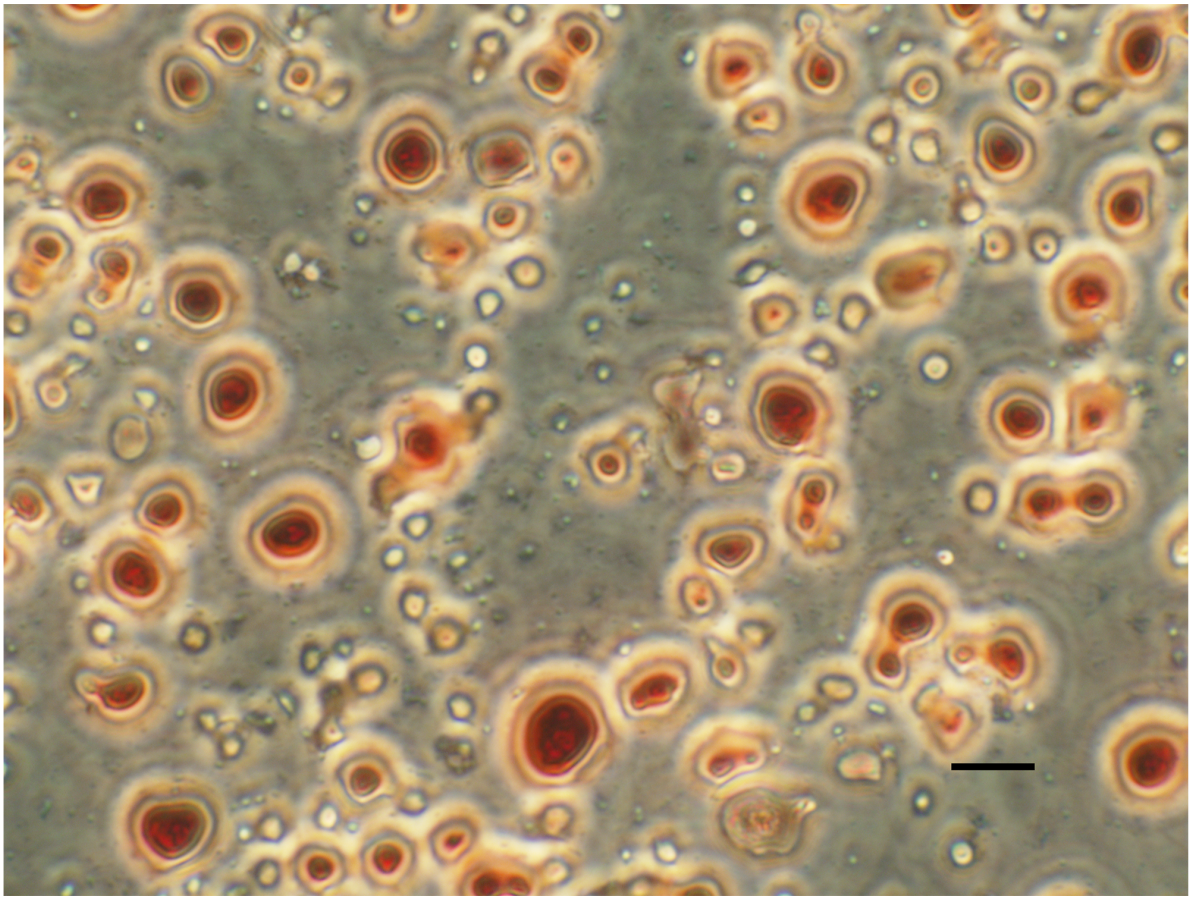

Figure 5.1 Adipocytes Under Light Microscopy After Staining with Oil Red O

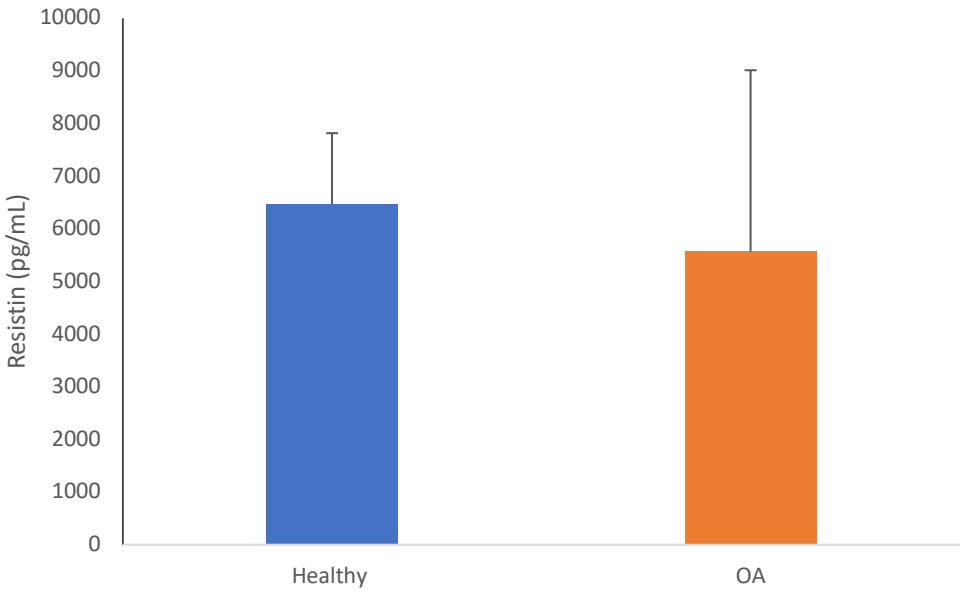

Figure 5.2 Mean (± SEM) synovial fluid resistin concentrations in dogs with osteoarthritis (OA) and normal stifles. Student's t-test was done for statistical analysis, $p = 0.076$.

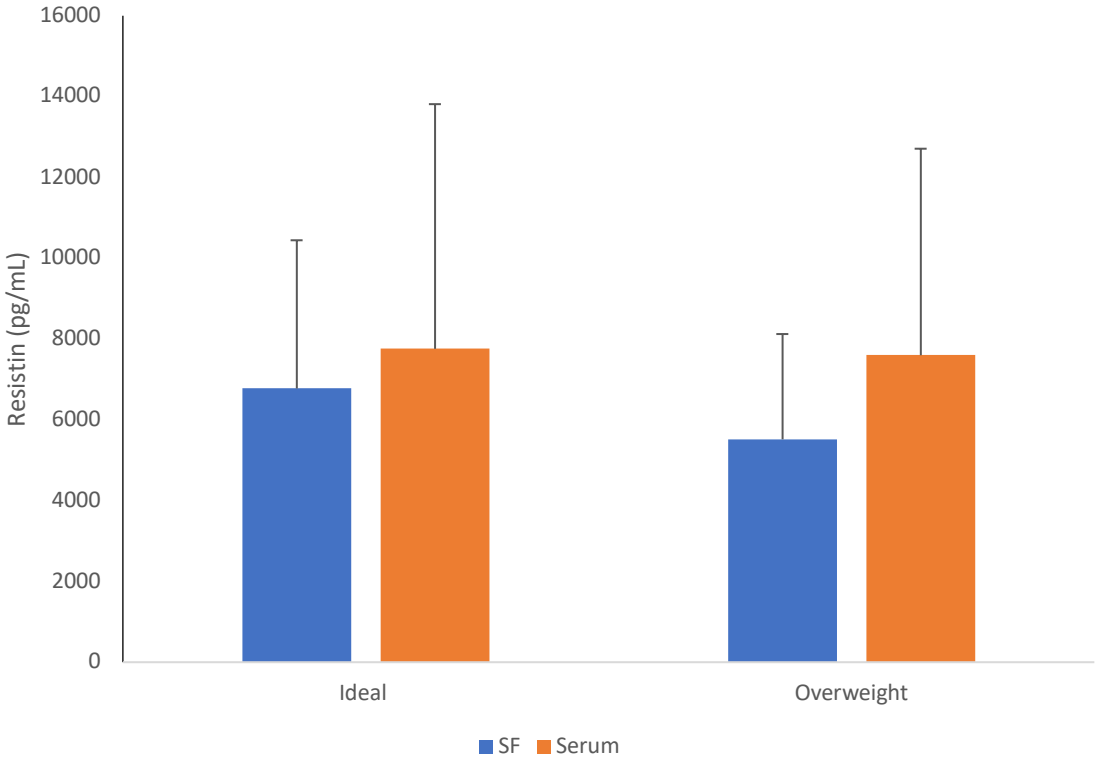

Figure 5.3 Mean (± SEM) serum and synovial fluid resistin concentrations based on body condition score. Student's t test was done for statistical analysis, p = 0.11for serum and p = 0.23 for synovial fluid

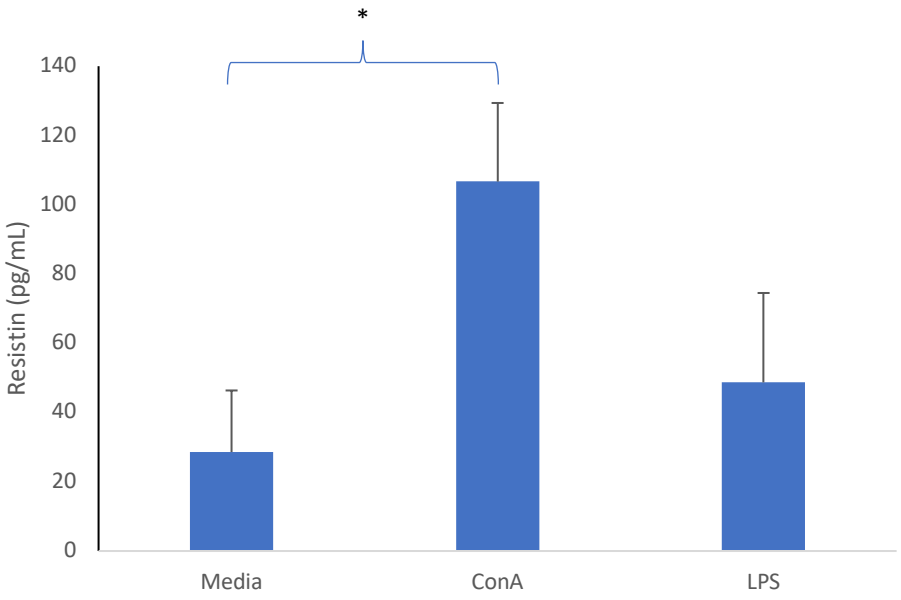

Figure 5.4 Mean (± SEM) resistin concentrations in peripheral blood mononuclear cell culture supernatant from 6 healthy dogs. Peripheral blood mononuclear cells (PBMCs) were cultured under 3 conditions – media alone, concanavalin A (Con A) 1 µg/mL, or lipopolysaccharide 1000 µg/mL. (PBMCs) were cultured with treatments for 24 hours. A linear mixed effects model was used for statistical analysis. Tukey's test was used for multiple comparisons. *= Significantly different from media; p = 0.047.

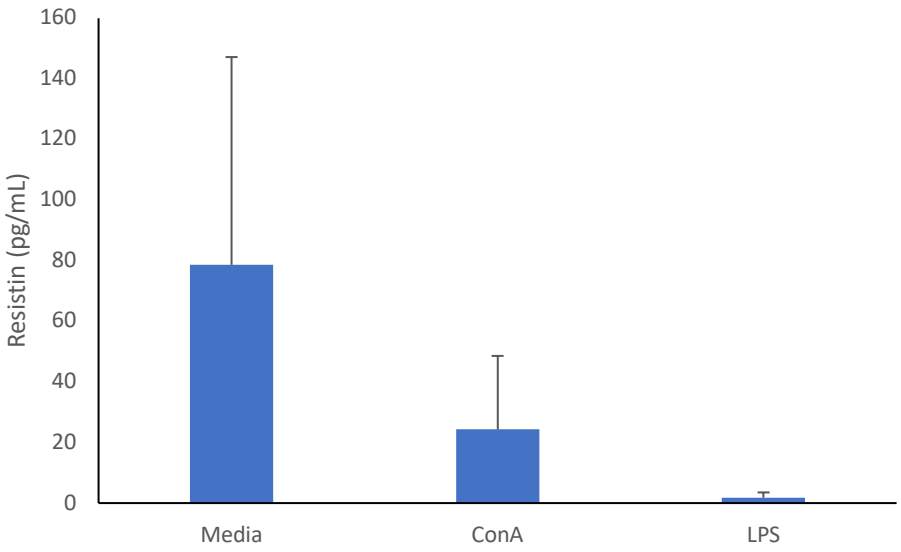

Figure 5 Mean (± SEM) resistin concentrations in canine adipocyte culture. Perigonadal white adipose tissue was harvested from 6 healthy dogs at the time of sterilization. Pre-adipocytes were differentiated over 12 days into mature adipocytes. Mature adipocytes were cultured for 24 hours under 3 treatment conditions, media alone, Concanavalin A (Con A) 1 µg/mL, or lipopolysaccharide (LPS) 1000 µg/mL; Repeated measures ANOVA was used to test for differences amongst treatments, $p = 0.42$.

CHAPTER 6

CONCLUSION

Osteoarthritis (OA) is a highly prevalent condition and is a major source of pain, decreased mobility, and decreased quality of life in the canine population. Obesity is also a common comorbidity in veterinary medicine and has been linked to OA. While there are biomechanical changes within the joint of obese dogs, biomechanics alone may not explain the link between OA and obesity. Therefore, the overall objective of the study was to evaluate the role of synovial fluid adipocytokines, specifically resistin and leptin, in a naturally-occurring model of canine osteoarthritis.

In the first experiment, we aimed to quantify synovial fluid leptin and correlate it with measures of body habitus, i.e. body condition score (BCS) and body fat percentage. It was hypothesized that synovial fluid leptin would be higher in overweight dogs compared to those that were ideal weight and that synovial fluid leptin would positively correlate with serum leptin and body fat percentage. The results presented in Chapter 3 confirm our hypothesis. While synovial fluid leptin correlated strongly with serum leptin, concentrations of leptin were significantly lower in synovial fluid when compared to serum. Additionally, synovial fluid was compared between dogs with OA secondary to cranial cruciate ligament injury and those with no evidence of joint disease. In the OA group, associations between synovial fluid leptin and radiographic severity of OA,

caregiver perceived pain and joint dysfunction, synovial fluid IL-1β, and dietary Ω-6: Ω-3 were investigated. The second hypothesis was that synovial fluid leptin would be higher in the OA group than in the healthy control group and that synovial fluid leptin would correlate with radiographic severity, joint pain and dysfunction as measured by the Liverpool Osteoarthritis in Dogs (LOAD), a previously validated clinical metrology instrument, and synovial fluid IL-1β. The second hypothesis was rejected as there was no difference in synovial leptin between OA and healthy control populations. Additionally, there was no correlation between synovial fluid leptin and radiographic severity of OA or IL-1β mediated synovial inflammation. Interestingly, there was a weak positive correlation between LOAD score and synovial fluid leptin and dietary Ω-6: Ω-3.

Our second goal was to evaluate the correlation between synovial fluid cytokines and chemokines and synovial fluid adipocytokines in dogs with secondary OA and those with healthy joints. This was accomplished by measuring synovial concentrations of the adipocytokines, resistin and leptin, via canine specific ELISAs and synovial fluid and serum concentrations of 13 chemokines and cytokines with a magnetic bead assay. While there was no association found between synovial fluid adipocytokines and granulocyte-macrophage colony-stimulating factor, interferon-γ, IL-2, Il-6, IL-7, IL-8, IL-10, IL-15, IL-18, IP-10, KC-like, monocyte chemoattractant protein 1 (MCP-1), or tumor necrosis factor α, higher concentrations of synovial fluid MCP-1, IL-8, and IL-6 were found in dogs with OA compared to healthy dogs. However, none of these cytokines were correlated with radiographic severity of OA, dietary Ω-6: Ω-3, or LOAD. Despite it not being the primary goal of the study, serum concentrations of IL-6 were found to be higher in overweight dogs when compared to ideal weight dogs.

Our final aim was to quantify resistin in the synovial fluid and compare it to OA status and measures of body habitus in the dog. Our first hypothesis in Chapter 5 was that neither synovial fluid nor serum resistin would be different in dogs that are overweight versus dogs that are in ideal body condition. Additionally, we believed it would be higher in synovial fluid of dogs with OA compared to healthy dogs. Consistent with one other canine study, serum and synovial fluid resistin was not higher in overweight dogs when compared to ideal weight dogs and there was no correlation with body fat percentage in dogs.

As resistin is released by monocyte/macrophage cell lines in humans and has been shown to not correlate with BCS in the canine, a secondary, yet novel, goal of Chapter 5 was to investigate the release of resistin from canine peripheral blood mononuclear cells and white adipose tissue derived adipocytes to elucidate whether resistin is truly an adipocytokine in the canine. We hypothesized that resistin would be released from monocytes but not adipocytes in culture. While there was no resistin release from canine adipocytes stimulated with Concanavalin A (a T cell mitogen) or lipopolysaccharide (LPS; a monocyte/macrophage stimulant), resistin was shown to be released from Concanavalin A activated peripheral T cells.

The results of the studies reported here indicate that the adipocytokines, leptin and resistin, are not elevated locally in the synovial fluid of dogs with OA. In addition, there was no correlation with either adipocytokine and radiographic score. This was not surprising given that to the authors knowledge there are no currently published reports of cytokines, chemokines, or other biomarkers being associated with radiographic severity of OA in the canine. While synovial fluid leptin, but not resistin, was weakly correlated

with LOAD, a biomechanical effect on caregiver perceived pain and joint dysfunction cannot be eliminated. As synovial fluid leptin was correlated with BCS and body fat percentage, it is likely that synovial fluid leptin is merely a reflection of systemic leptin. This leads to the possibility that synovial fluid leptin is higher with obesity and obesity is imparting a biomechanical effect on the joint, resulting in higher LOAD score.

An interesting finding in this study was that synovial fluid leptin was correlated with dietary Ω-6: Ω-3. In canines with OA, diets with a low Ω-6: Ω-3 have been shown to objectively improve lameness, likely through the proposed anti-inflammatory effects of Ω-3 fatty acids. In humans, diets rich in Ω-3 fatty acids may decrease systemic inflammatory mediators, including serum leptin. Because of these findings, further research into the effects of diet, particularly polyunsaturated fatty acids, on canine adipocytokines both locally and systemically is warranted.

Despite the evidence presented here, a role for adipocytokines in canine OA cannot be ruled out. First, the direct effects of neither leptin nor resistin on cartilage or the synovium was evaluated. It is possible that these adipocytokines modulate OA in a mechanistically different pathway than through the inflammatory cascade. In addition, matrix metalloproteinases (MMP), especially MMP-1, -3, and -13 were not examined in the synovial fluid. As they are important in cartilage extracellular matrix regulation through their collagenase activity, they may be modulated by local concentrations of adipocytokines.

As expected both synovial fluid and serum leptin was correlated with measures of body habitus in the canine. Also, there was a strong correlation between synovial fluid

and serum leptin. Synovial fluid is an ultrafiltrate of plasma; therefore, it is unclear as to whether synovial fluid leptin merely reflects systemic leptin or if there is local release of leptin from the infrapatellar fat pad. Consistent with previous canine work there was no association between either serum or synovial fluid resistin and BCS in the dog. Because of this, the release of resistin from peripheral blood mononuclear cells (PBMC) was examined. Surprisingly, activated T cells were the main source of resistin release in PBMC cell culture. There was no significant release of resistin from canine adipocytes in our model. However, it is unclear if the stimulus used to elicit resistin release in adipocytes was appropriate. It is possible that resistin is released from adipocytes under control of a different stimulant. However, in previous work done with canine adipocytes, LPS was capable of eliciting increased mRNA expression and protein release of the adipocytokines, leptin and adiponectin. Therefore, LPS was deemed appropriate for our model. While early studies suggest that resistin may not be an adipocytokine in the dog, further work is needed to not only determine the main source of resistin but also to elucidate the true physiologic role of resistin in the canine.

Finally, higher concentrations of IL-6 have been reported in 2 canine studies comparing OA to healthy synovial fluid. In addition, IL-6 has been correlated with proteoglycan synthesis, a marker of cartilage turnover. We are the first to report that both IL-8 and MCP-1 are higher in OA compared to healthy synovial fluid. The role of these chemokines in the pathophysiology of OA is unknown. MCP-1 is important for monocyte infiltration and chemotaxis. Given the increase in mononuclear cells in OA synovium, MCP-1 may play a role in this chemotaxis. This has been demonstrated in a rodent model of OA. On the other hand, IL-8 is chemotactic for neutrophils, which are rarely present in

significant numbers in the OA joint. Therefore, the role of IL-8 in OA is perplexing. At least one human study, has linked both of these chemokines to the OA associated pain severity but no currently published canine studies exist. However, there was no correlation between either IL-8 or MCP-1 and LOAD. Further studies are warranted to examine the role of these chemokines in OA pathophysiology.

The data presented here highlights the presence of both leptin and resistin with canine synovial fluid. Even though neither adipokine was correlated with OA status, the findings here highlight the importance of weight management in the treatment of OA associated pain and joint dysfunction. In addition, this work highlights a potential role for resistin in modulation of either immune or inflammatory pathways, as it is released from active T cells. Still, further studies are necessary to examine canine adipocytokines and their role in local and systemic inflammatory conditions.

APPENDIX A

SYNOVIAL MEMBRANE RECEPTORS AS THERAPEUTIC TARGETS: A REVIEW OF RECEPTOR LOCALIZATION, STRUCTURE, AND FUNCTION

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A.1 Abstract

Joint pathology and degeneration is a significant cause of pain. The synovial membrane plays an important role in maintenance of the joint, contributes to the pathology of many arthropathies and may be adversely affected in joint disease. Improving knowledge of the receptors present within the synovium will aid in a better understanding of joint pathology and the development of new treatments for diseases such as osteoarthritis and rheumatoid arthritis. Knowledge of the location and function of synovial membrane receptors (both in healthy and diseased synovium) may provide important targets in the treatment of various arthropathies. Classic pain receptors such as opioid receptors in the synovium are a mainstay in local and systemic management of chronic pain in many species. In addition to these, many other receptors such as bradykinin, neurokinin, transient receptor potential vanilloid, and inflammatory receptors, such as prostanoid and interleukin receptors have been discovered within the synovial membrane. These receptors are important in pain, inflammation, and in maintenance of normal joint function and may serve as targets for pharmacologic intervention in pathologic states. The goal of this review is to outline synovial membrane receptor localization and local therapeutic modulation of these receptors, in order to stimulate further research into pharmacological management of arthropathies at the local level.

Keywords: Synovial membrane, Receptor, Arthropathy, Intra-articular

A.2 Introduction

Arthropathies can be a significant source of pain and finding new therapeutic treatments to alleviate that pain is paramount. The synovial membrane plays an important role in maintenance of the joint, contributes to the pathology of many arthropathies [1] and may be adversely affected in joint disease [2]. The synovial membrane consists of two layers, the intima and the subintima, with a small amount of hyaluronan between layers. The intima is relatively acellular but consists of 2 types of synoviocytes, type A, which is of macrophage or monocyte cell lines and type B, which is a fibroblast. The subintima is composed of blood and lymphatic vessels [3] and loose connective tissue and merges with the outer layer of the joint capsule [1].

The synoviocytes are important in producing synovial fluid for cartilage lubrication, chondrocyte nutrition, and control of synovial fluid volume through the production of hyaluronic acid [1,3]. They may also play an important role in joint pathology by increasing joint friction, regulation of inflammation and fibrosis [1] and regulation of synovial immune function [3]. Synovial inflammation is evident in osteoarthritis (OA) and inflamed synoviocytes may react to cartilage degradation enzymes and result in the release of cytokines and matrix metalloproteinases [2,4].

Knowledge of the receptors present within the synovium, may aid in the understanding of joint disease and lead to development of new treatments for arthropathies. Extensive research has determined many of the important receptors responsible for normal synovial membrane function and those important in modulating disease, particularly inflammatory arthropathies. The presence and function of synovial

membrane receptors in both normal and diseased synovium are an important target in the prevention and treatment of arthropathies and will be discussed in this review.

A.3 Receptor Structure and Function

Opioid Receptors

Endogenous opioids, enkephalin, endorphins, and dynorphin, are primarily involved in anti-nociception [5], although nociceptin can display either pro- or anti-nociceptive characteristics [6]. The opioid receptors are G-protein coupled receptors and include the μ, κ, δ, and nociceptin receptor types [7]. Synovial opioid receptors have been demonstrated in humans [8-11], dogs [12], rats [13], and horses [14,15]. μ- opioid receptors have been detected in the synovium of normal rats[13] and humans with OA, rheumatoid arthritis (RA), and joint trauma [9], while κ- and δ- receptors have been demonstrated in the RA and OA synovium [10].

While opioid receptors are known to be important in analgesia, they may also play a role in inflammation as outlined in Table 1. The density of these receptors is significantly increased in inflamed joints [12,15] compared to healthy joints [10,16]. However, receptor down-regulation may occur in chronic arthropathies [8,10]. In patients with chronic OA, the expression of δ- and κ- opioid receptors is significantly reduced compared to normal expression [10].

Glucocorticoid Receptors

Glucocorticoid receptors are cytoplasmic receptors that upon binding of ligand, i.e. cortisol, translocate into the nucleus, where this receptor-ligand complex is able to alter transcription activity of many genes. This can result in changes in glucose metabolism, cell differentiation, and can modulate the inflammatory response [17]. Glucocorticoid receptors have been identified in synovial tissue from RA, OA [18-20], and traumatic arthritis patients, with no difference in density among groups [19,20]. These receptors are primarily localized in inflammatory lymphoid cells and C reactive protein level was correlated with the density of cells expressing glucocorticoid receptors [20]. Activation of glucocorticoid receptors prior to treatment with TNFα decreased IL-1β in cultured synovial fibroblasts[21]. While glucocorticoids are often used as anti-inflammatories and analgesics, there seems to be no correlation between the density of glucocorticoid receptors and pain [18].

Glutamate Receptors

Glutamate is an excitatory neurotransmitter within the central nervous system [22], particularly the dorsal root ganglia of the spinal cord [23] and is important in nociception. There are two main types of glutamate receptors, metabotropic (mGluR) and ionotropic (iGluR). The iGluRs include NMDA, AMPA, and kainate[24]. Increased synovial fluid glutamate has been detected in patients with arthropathies [25], leading to the evaluation of glutamate receptors in the synovium.

The NMDA receptor is important in nociceptive modulation [26] and the development of central sensitization. Activation results in excitation of sensory neurons

or secretion of inflammatory peptides [27]. NMDA receptors are comprised of two NR1 subunits and any combination of two NR2 subunits, NR2A-D [27,28]. There is evidence for the presence of the NR2D subunit in the healthy rat synovium [29] and both the NR1 and NR2 subunits in human synoviocytes [27]. In rat synovium, iGluR3 and kainate2 mRNA was detected but no other subtypes were detected[29]. In a different study, kainate1 and iGluR2 receptors were identified in both rat and human synovium[30].

Transient Receptor Potential Channels

The transient receptor potential (TRP) receptors are ion channels that detect and transduce noxious and non-noxious thermal and chemical stimuli. Since they act as nociceptors, they may also be involved in pathological pain states [31]. There are seven members of this family including TRP vanilloid (TRPV), TRP ankyrin (TRPA), TRP melastatin (TRPM), and TRP canonical (TRPC). TRPV1 detects noxious thermal signals (>42°C) [32]. Activation of TRPV1 has resulted in induction of the formation of the metabolites of unstable reactive oxygen species [33], and increased the expression of cyclooxygenase-2 [34], TNFα, and the chemokine RANTES [33,34]. TRPV1 receptors were detected on synovial membranes of human synoviocytes[35] and in the synovial membrane of rat temporomandibular joints [36].

TRPV3 and TRPV4 have both been detected in healthy synoviocytes [35]. Due to the lack of receptor activation in the TRPV3 temperature range [37], it is likely that TRPV4 is the predominant “moderate” temperature receptor in the synovium. TRPM3, 7, and 8 have also been detected in the synovium [32,38,39]. TRPM3 is primarily activated by hypotonic cellular swelling[38] and TRPM8 responds to cool temperatures (less than

25°C) [39]. The expression of functional TRMP3 has been detected in healthy rabbit synovium. TRPM7 was detected in the synovium of rats with adjuvant-induced arthritis and healthy rats with higher expression in the arthritic synovium [40].

TRPA1 is a cold sensing ion channel that has been detected on nociceptors and may play a role in hyperalgesia [41] and cold hypersensitivity [42]. TRPA1 was detected in healthy and adjuvant-induced arthritis mouse synovial membrane [43] and appears to mediate increases in blood flow to arthritic joints in response to cold exposure [43]. Finally, TRPC5, which is activated by thioredoxin, has been found in like fibroblast-like synoviocytes from RA patients [44,45]. TRCP5 has been associated with a decrease in synovial fluid cytokines, in mice [45]. Despite their synovial localization, the function of the TRP receptors in both synovial thermal nociception and inflammation is not well defined.

Chemokines

Chemokines are small, secreted molecules that are important in the promotion of inflammation, angiogenesis, chemotaxis, and cartilage degeneration [46,47]. There are greater than 50 known chemokines that bind to 20 different G-protein coupled receptors [47]. They are divided into $CCLR_{1\text{-}11}$, XCR_1, $CXCR_{1\text{-}7}$, and CX_3R_1 [47,48].

Chemokines have been discovered within the synovial fluid from humans with RA, OA, and joint trauma [49-52] and in healthy horses and horses with osteochondral injury [53]. In rats with adjuvant-induced arthritis [54] and humans [48], CCR1, CCR4, CCR5 [48,54] and CCR2 [48] were identified in synovial tissue, with higher expression of CCR

in OA joints compared to healthy joints [55]. CXCR1, CXCR2, and CXCR4 have been detected in the synovium of rats [48] and CCR9 in RA and OA synovial tissue [47].

CCR1, CCR2, and CCR5 expression in the synovium has been positively correlated with the peak of clinical inflammation and is associated with macrophage and lymphocyte infiltration [46]. Synovial CCR9 has also been implicated in mononuclear cell infiltration [47], while CXCR1 and CXCR2 are involved in neutrophil chemotaxis [56] Additionally, CCL2 has been shown to increase monocyte adhesion to synoviocytes via CCR2 activation [46]. Blockade of CCR9 decreased matrix metalloproteinase-3 related cartilage degradation [47]. CXCR3 may mediate fibroblast invasion, which may be important in the development of arthropathies [57,58].

Interleukin Receptors

Interleukins (IL), a member of the cytokine family, are important in modulation of the inflammatory response. They have been found in synovial fluid from patients with arthropathies [59]. Members of the IL-1 family of receptors that have been detected in the synovium include IL-1R and IL-18R [60]. Receptors belonging to the IL-17R family have been detected in the synovial intimal lining in patients with RA, OA [61,62], psoriatic arthritis, and traumatic arthropathies [62]. In RA synovium, a correlation between IL-1R expression and IL-6, IL-8, PGE_2, and vascular endothelial growth factor release may point to a role for IL-1R in cytokine release [63].

IL-18R [64,65] and IL-21R [66] have been detected in the synovial membrane of patients with RA. While some OA tissue but not all expressed IL-18R [64] and none expressed IL-21R [66]. These receptors and chemokines may be important not only in

inflammation but also in synovial hyperplasia, as IL-22 increased synoviocyte proliferation in both RA and OA tissue [67].

Prostanoid Receptors

Prostanoids are important in inflammation particularly in mediating pain, swelling, vasodilation, and vascular permeability. The prostanoid receptors are G-protein coupled and there are 4 subtypes of PGE receptors, EP1-4 [68]. In humans, synovial localization of EP2 and EP4 was confirmed, while EP1 and EP3 were not detected [69]. In rats with adjuvant-induced arthritis, EP2 [70], EP3B [70,71], and EP4 [70,72] are expressed [70,71].

In rat synovial cells, EP2 and EP4 agonists attenuated IL-1 induced IL-6 expression and release [70], while EP3 selective agonists have resulted in increased IL-6 production [71]. In human OA and rat synoviocytes these agonists decreased IL-1 induced monocyte chemoattractant protein-1 production [73]. Finally, blockade of EP4 resulted in a significant reduction of arthritis scores in mice [74] and activation of EP2 in rabbits has been associated with increased type II collagen and cartilage regeneration [75].

Prostacyclin, another prostanoid, is systemically important in platelet activation and vasodilation. Both prostacyclin and its receptor are constitutively expressed by OA synoviocytes [76]. Inhibition of the prostacyclin receptor in the mouse synovium resulted in significantly lower subjective arthritis scores, less inflammatory cell infiltration, less bone and cartilage destruction, and decreased IL-1β and IL-6 production [74].

Histamine

Systemic histamine is functionally important for allergic reactions, inflammation, and gastric acid secretion [77]. The effects of histamine are mediated through 4 distinct G-protein coupled receptors, H_1R, H_2R, H_3R, and H_4R [78-80]. H_1R and H_4R have been variably expressed in the OA synovium [78,80].

Histamine has resulted in increased expression of IL-1β, IL-6, and IL-8 in synovial cell cultures [79]. Systemic blockade of H_4R attenuated the release of synovial TNF-α in response to intra-articular administration of lipopolysaccharide [81]. A negative correlation has been identified between serum matrix metalloproteinase-3 concentration and synovial H_4R expression [80], which may represent a negative feedback loop in synovial inflammation.

Bradykinin

Bradykinin is a mediator of inflammation that can cause smooth contraction, vasodilation, increased vascular permeability, increased leukocyte migration, and induction of pain [82]. It is also up-regulated in the presence of inflammation [83]. Bradykinin binds to 2 different receptors inducible B_1 and constitutive B_2 [84]. As early as 1992, bradykinin receptors were detected in synovial joints [82,83]. The B_2 receptor was found in healthy and inflamed synovial tissue in rats [85] and the synovial lining of human OA tissue [84]. B_1 expression was only present in some rats after induction of arthritis[85] and none of the human OA samples [84]. Patient selection may have prevented the detection of B_1 receptors as the majority of patients had chronic disease and B_1 is likely induced during acute inflammation.

In rabbit knees activation of B_1 was associated with an increase in synovial blood flow within the inflamed joint [86]. Through the actions of B_2, bradykinin can increase the production of IL-1 [87,88] and IL-8 in the synovium, independent of cyclooxygenase [87]. Finally, B_2 activation has been associated with local endothelial cell proliferation in mildly inflamed joints [85].

Tachykinin

Neurokinin receptors are G-protein coupled and bind primarily to tachykinins [89]. Substance P primarily binds to the neurokinin 1 receptor, NK_1 [90] and stimulates angiogenesis, up-regulates pro-inflammatory cytokines [91], causes vasodilation, and increases vascular permeability [92].

Substance P has been determined to be present in human OA joints [93,94] and in lipopolysaccharide inflamed equine joints [95]. NK_1 receptors have been identified in synoviocytes from patients with OA [91] and in rat synoviocytes [96]. The role of these receptors in the synovium, to the authors' knowledge, has not been examined.

Serotonin

Serotonin, 5-HT, has been implicated in spinal mediated anti-nociception through excitation of inhibitory interneurons within the spinal cord. 5-HT may also be involved in spinal facilitation of descending nociceptive signals, making it both pro- and anti-nociceptive. There are several 5-HT receptor subtypes, 1_{A-F}, 2_{A-C}, $3,4,5_{A,B}$, 6,7. All of which are metabotropic, with the exception of $5\text{-}HT_3$, which is ionotropic [5].

A study in rats with adjuvant-induced arthritis, identified elevated levels of synovial fluid 5-HT in inflamed compared to healthy joints [97]. 5-HT_{1A}, 5-HT_{1B} [98], 5-HT_{2A} and 5-HT_3 [98,99] receptors have been detected in OA synovial tissue [100,101]. In synoviocytes, 5-HT results in an increase in the expression of PGE_2 [99]. The effect on PGE_2 release was blocked by the 5-HT_2 antagonist, ketanserin [101].

Purinergic Receptors

The purine receptors are divided into the P1, P2, and adenosine types. The adenosine receptor is comprised of 4 distinct subtypes $Adenosine_1$, $Adenosine_{2A}$, $Adenosine_{2B}$, and $Adenosine_3$, which are all G-protein coupled and are anti-inflammatory. The main ligand for adenosine receptors is adenosine [102-104]. The P2X type of P2 receptors is a ligand-gated ion channel [104]and is further divided into 7 subtypes, $P2X_{1-7.}$ Signaling is mediated by binding of ATP [105]and can result in either pro- or anti-inflammatory actions. Adenosine has been identified in the synovial fluid from humans with OA and RA [106]. OA synoviocytes expressed $Adenosine_1$, $Adenosine_{2A}$, $Adenosine_{2B}$, and $Adenosine_3$ receptors with higher expression of $Adenosine_{2A}$ and $Adenosine_3$ [104]. In bovine synoviocytes, $Adenosine_1$, $Adenosine_{2A}$, $Adenosine_{2B}$, and $Adenosine_3$ receptors were also identified [102,103].

Adenosine and its receptors are associated with decreases in inflammation [102-104]and decreased cartilage destruction [107,108]. Lipopolysaccharide induced release of PGE_2 is inhibited by administration of $Adenosine_1$, $Adenosine_{2A}$, $Adenosine_{2B}$, and $Adenosine_3$ agonists in bovine synoviocytes [103], while administration of $Adenosine_{2A}$

and Adenosine$_3$ agonists resulted in decreased release of IL-8 and TNF-α in response to lipopolysaccharide in end stage OA synovium [104].

P2 receptors have also been identified in synovial tissue [104,105,109]. In human OA synoviocytes, mRNA expression of P2X$_{1-7}$ was seen, with higher expression of P2X$_1$, P2X$_3$, and P2X$_7$ [104]. P2X$_1$ resulted in decreased production and release of TNF-α in the OA synovium [104], while both P2X$_3$ [104] and P2X$_4$ agonists increase the release of TNF-α. P2X$_4$ activation also increases IL-1, IL-6, IL-17 [110] and brain derived neurotrophic factor, while inhibition is associated with decreased inflammatory cell infiltration and synovial hyperplasia [40]. P2X$_7$ agonists also increase synovial IL-6 production [105].

Lipoxin

Lipoxins are lipid inflammatory mediators, formed from arachidonic acid, that are found during the resolution of inflammation [111]. ALX is the receptor for lipoxin A4; however, serum amyloid A also appears to be an important ligand for this G-protein coupled receptor [111,112]. ALX expression has been identified in human synoviocytes, and the expression is increased in the presence of IL-1β [112].

Lipoxin A$_4$ is important in the inhibition of neutrophil activation and can alter the release of pro-inflammatory cytokine [111,113]. Synovial lipoxin A$_4$ is associated with the release of IL-6, IL-8, and matrix metalloproteinase-3 in response to IL-1β [113]. Finally, in human synoviocytes, ALX ligands can decrease serum amyloid A and inflammatory cell chemotaxis and induce IL-8 release [112].

Non – Neuronal Cholinergic System

Acetylcholine is a neurotransmitter that binds to 2 different cholinergic receptors, nicotinic and muscarinic. Nicotinic receptors are inotropic and are typically sodium channels. Degradation of acetylcholine is due to activity of acetylcholinesterase. The nicotinic receptors, particularly the α7 receptor (α7R), modulate inflammation by decreasing the synthesis of cytokines [114] and are present in a variety of tissues such as immune and inflammatory cells [115]. The α2, α3, α4, α5, α6, α7, α9, and α10 subunits of the nicotinic receptor were identified in OA synovium [116]. The α7R has also been noted in the synovium from healthy volunteers [117]. In the synovium, acetylcholine and pyridostigmine, an acetylcholinesterase inhibitor, decrease IL-1 induced IL-6 release [115].

Epidermal Growth Factor Receptor

Epidermal growth factor receptor (EGFR) is a member of the family of receptor tyrosine kinases. This family of receptors plays a major role in cellular proliferation and cell maintenance. The ligands for EGFR are transforming growth factor-α, amphiregulin, and EGF [118]. Several ligands for EGFR, particularly amphiregulin, have been identified in synovial tissue of RA and OA patients, with higher expression in RA [119]. In RA synovial tissue, amphiregulin results in increased VEGF, IL-8, and IL-6 expression. Additionally, expression of EGFR has been detected in the synovial lining of patients with RA and mice with adjuvant-induced arthritis [120]. In a mouse model of RA, administration of an EGFR auto-inhibitor decreased arthritis scores [121]. While the role of EGFR has not been as extensively evaluated in OA, mice with decreased EGFR

activity have a slower progression of post-traumatic OA and less cartilage damage than those with normal EGFR activity [122]. Based on the results of these studies, EGFR signaling may be important in the development and progression of arthritis, however further studies are warranted.

Platelet-Derived Growth Factor Receptor

Another receptor tyrosine kinase, platelet-derived growth factor receptor (PDGFR), has PDGF as its main ligand. Several isoforms, A-D, of PDGF exist, with high expression of PDGF- B, moderate expression of PDGF-C and –D, and low expression of PDGF-A in OA and RA synoviocytes [123]. Systemic expression of PDGFR is low but is induced in a variety of tissues during inflammation [124]. An early study evaluating human synovial expression of PDGFR, determined that PDGFR was not expressed in the healthy synovium; however, expression was seen in inflamed synovial tissue [125]. In RA synovial tissue, activation of PDGFR promotes the formation of invadosomes, which are vesicles in arthritic synoviocytes that promote extracellular matrix degradation [126]. Further studies are needed to fully understand this receptor's role in inflammatory arthropathies.

Vascular Endothelial Growth Factor Receptor

Vascular endothelial growth factor receptor (VEGFR), a receptor tyrosine kinase, signals via VEGF through VEGFR1 and 2 [127]. VEGF is important in angiogenesis and may play a role in inflammation. Blockade of VEGF in mice with adjuvant-induced arthritis delayed the onset of arthritis, decreased histologic evidence of synovitis and joint destruction, and decreased vascular density [128]. VEGFR1 has been detected in

synovial tissue of patients with temporomandibular joint derangement, control joints [129], in patients undergoing hip arthroplasty [127], and in healthy rabbits and those with experimental temporomandibular trauma [130]. Although VEGF and its receptor are important in angiogenesis, the role of VEGF in OA and RA warrant further study.

Fibroblast Growth Factor Receptor

Fibroblast growth factor receptor (FGFR) is another member of the receptor tyrosine kinase inhibitor family. FGF signals through 4 receptor variants FGFR1-4 [131]. Like VEGFR1, FGFR-1 has been detected in synovial tissue of patients with temporomandibular joint derangement, control joints [129], in patients undergoing hip arthroplasty [127], and in healthy rabbits and those with experimental temporomandibular trauma [130]. The effects of FGF on cartilage homeostasis are controversial, as some studies report catabolic effects [132], while others report anabolic effects [133]. These effects have been further classified and appear to be species dependent. In mice with meniscal injury, FGF prevents progression of OA, while in human articular cartilage, FGF results in proteoglycan loss. This is suspected to be due to differential expression of FGFR subtypes, as mice primarily express FGFR2 and FGF4, which is anabolic, and catabolic FGFR1 and FGFR3 predominate in human cartilage [134]. Given the contrasting results, the role of FGFR within the synovium warrants further investigation.

Discoidin Domain Receptor 2

The final tyrosine kinase receptor evaluated in the joint is discoidin domain receptor 2, which is expressed in the synovial lining in OA tissues [135] and has a role in cartilage

maintenance and pathologic destruction [136]. Further studies regarding the effects of these receptors within the synovium are warranted.

Toll Like Receptors

TLRs are a group of pattern recognition receptors that are important in the recognition of microbial components and endogenous inflammatory mediators. There are 10 types of TLRs reported in mammals, TLR1-10 [137]. In synovial tissue from healthy and OA joints, TLR1-6 receptors are expressed, with TLR3 followed by TLR4 showing the highest expression, and TLR5 with extremely low expression [138]. In another study, TLR2 and TLR5 expression was identified in both OA and normal synoviocytes, with greater expression in OA tissue [139]. TLR2 and 4 have been detected in the synovium of dogs with OA [140] and TLR7 has been identified in the synovial lining of rats with adjuvant-induced arthritis [141].

Synovial TLR seems to play a significant role in synovial inflammation and the development of inflammatory arthropathies, as inhibition of TLR7 expression decreases cartilage damage and the production of IL-6 and IL-1β in rats [141]. Synovial TLR 2, 3, 4 [142], and 5 [143] activation induces IL-6 and matrix metalloproteinase-3 release. Additionally, stimulation of TLR2 and 4 augment production of TNFα, IL-6, and IL-8, and may up-regulate matrix metalloproteinases-1, 2, 3, and 13 [144].

Bone Morphogenetic Protein Receptors

Bone morphogenetic proteins function as cytokines, growth factors, and also modulate bone metabolism. There are 2 types of bone morphogenetic receptors (BMPR), Type I and Type II, with several subtypes of the type I receptor, type IA, type IB, and

type IA activin receptor. BMPR-1A, BMPR-1B, and BMPR-2 were detected in the synovium [145,146], with significantly higher expression of BMPR-1A [146]. BMPR-1A synovial expression has been negatively correlated to histologic grade [145] and may be important in the production and expression of extracellular matrix in articular cartilage [147].

Adipocytokine Receptors

Adiponectin is an adipocytokine that has been shown to have insulin–sensitizing effects [148], and may aid in artherosclerosis regulation, and the induction of inflammation [149]. With the discovery of a link between adipocytokines and inflammatory joint disease [148], research into synovial adipokines was warranted. Adipocytokines have been identified in synovial fluid of both RA and OA patients [150]. Articular adiponectin has been linked to increased production of monocyte chemoattractant protein-1 [149], IL-6 [148,149], IL-8 [151], and PGE_2 [152] by synoviocytes, and has resulted in higher concentrations of synovial fluid IL-6 [149]. Adiponectin receptors 1 and 2 have been detected in synovial tissue [149,152] from humans with RA and OA [149], however the function of these receptors in the synovium is not known.

Lipoprotein Receptors

Lipoproteins, transporters of triglycerides, are important in inflammation. They have been associated with the progression of OA [153], therefore synovial lipoproteins and their receptors have been evaluated. Low- and high-density lipoprotein receptors have been discovered in RA synovium [154] but they have not been evaluated in OA tissue. By

blocking the high-density lipoprotein receptor, inhibition of serum amyloid A induced IL-6 and IL-8 release occurred [155].

Lysophosphatidic Acid

Lysophosphatidic acid is an active phospholipid that is important in angiogenesis and inflammation [156,157]. It is the ligand for 6 G-protein coupled receptors, LPA_{1-6} [157,158]. Expression of LPA_{1-3} [156,158] and LPA_{4-6} has been detected in human RA and OA synovial fibroblasts [158]. Lysophosphatidic acid binding to LPA_1 resulted in up-regulation of the expression of the adhesion molecules VCAM and ICAM [158]. LPA_1 blockade prior to induction of arthritis, results in a significant reduction in inflammation associated with adjuvant injection [157,158].

Endothelial Protein C Receptors

Endothelial Protein C receptor is important in coagulation but may also enhance anti-inflammatory action. It binds with similar affinity to both protein C and activated protein C. It is important in coagulation but may also enhance anti-inflammatory activity. It is primarily expressed on endothelial cells [159], but has been identified in the synovial lining of RA and OA tissue [160]. Its function in OA synovium is currently unknown.

Angiotensin II Receptors

Classically, angiotensin II is thought of as a regulatory enzyme involved in fluid balance and control of blood pressure, however there is some evidence it may be involved in up-regulation of pro-inflammatory cytokines [161]. Angiotensin II is formed

from angiotensin I, under the control of angiotensin converting enzyme. There are 2 types of angiotensin II receptors AT1R, which is responsible for a majority of the cardiovascular effects, and AT2R, which is up-regulated in inflammation, ischemia, and trauma [162].

Captopril, an angiotensin converting enzyme inhibitor, has shown therapeutic benefits in patients with RA, which led to the discovery of angiotensin converting enzyme within synovial fluid [163]. Both AT2R [162] and AT1R have been detected in rats with induced arthritis [161,162]. Also, AT1R has been identified within the RA and OA synovium [164], however the role in the synovium is not well studied.

Cannabinoid Receptors

The cannabinoids have received recent attention as potential analgesic drugs. There are 2 main types of cannabinoid receptors, CB1 and CB2, which are both G –protein coupled [165, 166]. Typically, the CB1 is more abundant in nervous tissue while CB2 is more localized in immune tissue [47,166,167]. Endocannabinoids have been detected in synovial tissue [165]and synovial fluid [168] from both healthy patients and those with active arthropathies. CB1 [165] and CB2 [169] receptors are expressed in the OA synovium. CB2 receptors have also been detected in synovial membranes of both healthy and rats with adjuvant-induced arthritis [170]. Further research into the analgesic effects of synovial cannabinoid receptors is warranted.

Estrogen Receptors

Estrogen receptors α and β are members of the steroid receptor family [171]. Due to the correlation of synovial fluid estrogen levels and arthritis severity [171], estrogen

receptors in synovial tissue were evaluated. In the synovium of healthy male rats [171,172] and those with induced arthritis [171], estrogen receptor α was localized within synoviocytes [172]. In human synovial tissue, both estrogen receptor α and β have been detected from healthy, RA [173], and OA samples [19]. Estrogen administration in rats has resulted in a dose-dependent decrease in cartilage thickness and a decrease in extracellular matrix [174].

Notch Receptors

Notch receptors are important in cell differentiation, cell homeostasis, apoptosis [175] and chondrogenesis [176]. Mammals have 4 distinct Notch receptors, Notch1-4, with 5 ligands. The ligands, like their receptors, are trans-membrane proteins, that activate the receptor during cell-to-cell contact [175]. Notch 1 and Notch 3 receptors have been localized to the synovial lining in RA tissue [177]. Synovial Notch1 receptors have also been found in 7-day old calves [178] and Notch 1, 2, and 4 in postnatal mice [179].

While these are found in developing animals [178,179]they do appear to have several functions in adult animals. In OA, inhibition of Notch signaling results in decreased proliferation of chondrocytes. Additionally, expression of insulin like growth factor 2 increased, while bone morphogenetic protein-2 and IL-8 expression were decreased [180]. Over-expression of Notch results in increased matrix metalloproteinase-13 levels and inhibition of Notch results in increased quantity of type II collagen [181].

Relaxin Receptor

Relaxin is important in modulating ligament laxity during pregnancy and has been recently implicated as a regulator of collagen metabolism. It has been detected in the

synovial lining of patients undergoing arthroplasty [182]. Despite its synovial localization, its role in the joint is unclear.

Death Receptor

A relative of the tumor necrosis factor receptor, the death receptor, is important for apoptosis and has been detected in mouse synovial tissue, after the induction of inflammation [183]. Inhibition of death receptor 3 has resulted in significantly less cartilage degradation and matrix metalloproteinase-9 levels [183]. Further studies on the role of this receptor in cartilage maintenance are warranted.

A.4 Therapeutic Modulation of Receptors in the Synovium

Opioid receptors

Peripheral opioids and their receptors have been shown to have anti-inflammatory effects [11]. Intra-articular administration of endorphin has resulted in decreased plasma extravasation of leukocytes and plasma proteins [184] (Table 2). In horses, intra-articular administration of morphine resulted in significantly decreased joint circumference, synovial amyloid A, and plasma white blood cell count compared to intravenous morphine, demonstrating a local anti-inflammatory effect [185]. Both intra-articular δ- and κ- receptor agonists have been associated with a decrease in bradykinin induced plasma extravasation [186]. Also, a κ- specific agonist decreased myeloperoxidase activity in rats [187].

Glucocorticoid Receptors

The therapeutic role of glucocorticoids in arthropathies has been extensively evaluated. In RA, oral prednisolone has resulted in an increase in functional activity, decrease in pain, and decreased radiographic progression of joint destruction when compared to patients on disease modifying anti-rheumatic drugs [188,189]. In contrast, oral prednisolone has not been efficacious in the treatment of OA [190]. To limit the side effects of systemic glucocorticoids, intra-articular administration has been evaluated in humans [191,192], mice [193], and horses [95]. In RA, intra-articular glucocorticoid injections are a mainstay of symptomatic treatment as they decrease synovitis, joint swelling, and discomfort [194]. In horses, intra-articular administration of triamcinolone was associated with a significant improvement in lameness [195]. Finally, intra-articular injection of triamcinolone decreased mechanical allodynia as assessed by von Frey mechanical testing in mice with meniscal injury [193]. Despite their widespread use, not all patients respond to intra-articular glucocorticoid injection. Patients with higher levels of synovial fluid VEGF concentrations and those with greater joint destruction seem to be less likely to respond to treatment with intra-articular glucocorticoids [196]. Similar to RA, intra-articular administration of corticosteroids provide pain relief and increase joint function in patients with OA [191,197,198] in some but not all patients. Despite improvement in pain, no improvement in ultrasound parameters of synovial inflammation has been shown after administration of intra-articular corticosteroids [192]. As glucocorticoids are important in symptomatic relief of arthropathies, there is an extensive body of literature available regarding their use, however this is beyond the scope of this review.

Despite the analgesic benefits, disease-modifying effects of glucocorticoids in RA and OA are incompletely studied. In equine cartilage explants, the effects of methylprednisone and glycosaminoglycan synthesis were evaluated. Methylprednisone decreased glycosaminoglycan synthesis, even at low doses [199]. Similarly, high doses of prednisone resulted in chondrocyte toxicity but had no effect on glycosaminoglycans in porcine cartilage [200]. In addition to the effects on cartilage break down, glucocorticoids may result in chondrocyte apoptosis [201]. Prior to administration of intra-articular glucocorticoids, effects on the cartilage must be considered and further research into disease-modifying and chondrotoxic effects of glucocorticoids is warranted.

Glutamate Receptors

The local effects of glutamate modulating drugs have been evaluated. The NMDA antagonists, ketamine and memantine, have been associated with decreased pain and increased weight bearing in rats [202]. Intra-articular injection of inotropic glutamate receptor antagonists may decrease pain in mice [203], while intra-articular blockade of Group I metabotropic receptors in rats resulted in a reduction in pain from inflammatory arthritis [204]. In contrast, weight bearing and pain behaviors have improved with activation of both Group II and III metabotropic glutamate receptor agonists [205]. Local blockade of synovial AMPA and kainate receptors has resulted in a decrease in meniscal IL-6 concentrations in rats [30].

Transient Receptor Potential

TRPVs may be important not only in thermosensing but also in nociception. Intra-articular administration of a TRPV1 antagonist decreased pain in rats [33,206]. Also,

intra-articular injection of a TRPV4 agonist resulted in an increase in plasma extravasation, increased myeloperoxidase activity, and decreased the pain threshold [207].

Interleukin Receptors

IL-1 and its receptors may play an early role in the development of arthritis, therefore they may represent an important therapeutic target [208]. In bovine articular cartilage explants, antagonism of the IL-1 receptor decreased glycosaminoglycan release [209]. Early intra-articular administration of anakinra, an IL-1R antagonist, after anterior cruciate ligament transection in mice has resulted in decreased cartilage degeneration and increased lubricin, a glycoprotein important for lubrication and chondroprotection [210]. After articular trauma, administration of an IL-1R antagonist reduced cartilage degeneration and synovitis severity in mice. Additionally, these findings were more pronounced when this molecule was administered intra-articularly compared to systemic administration [211]. Local treatment of arthropathies with IL-1R antagonists show promise; however, the side effects of these antagonists is yet to be determined.

Tachykinin

The neurokin$_1$ receptor in arthropathies may be important in analgesia and inflammation. A neurokinin$_1$ receptor antagonist administered intra-articularly decreased cartilage erosion and decreased mechanical [92] and thermal hyperalgesia [212]. No analgesic effects of a different neurokinin$_1$ antagonist were present in another study but it did decrease plasma extravasation and myeloperoxidase activity [213].

Serotonin

5-HT and its receptors may be important in nociception, as intra-articular injection of 5-HT caused an increase in spinal neuronal excitation, specifically as it relates to nociception [214,215]. Local injection of ketanserin [215], and methiothepin, a 5-HT_7 antagonist [214], both decreased spinal excitation.

Toll Like Receptors

Modulation of the TLR offers a promising modality for the treatment of arthropathies, particularly RA. Previously it has been reported that 5-HT receptor antagonists inhibit TLR 3, 7, 8, and 9, therefore the evaluation of a commonly used selective serotonin reuptake inhibitor, fluoxetine, was evaluated. In mice with adjuvant-induced arthritis, fluoxetine slowed the progression of disease. Additionally, fluoxetine reduces TLR3 induced IL-6 production in human RA synovial fibroblasts [216]. Although other systemic inhibitors of TLRs may be effective in RA, there is a concern that system TLR inhibition could lead to immunosuppression; therefore, intra-articular therapies are attractive. Lubricin can also bind to TLR 2, 4, and 5 in a dose dependent manner, which results in decreased cytokine secretion in human OA synovial fibroblasts. Additionally, lubricin administered intra-articularly to mice with meniscal injury decreased synovial inflammation, cytokine expression, and pain [139]. Local TLR inhibition may represent a new target in the management of arthropathies.

Angiotensin II Receptors

Losartan, an angiotensin II receptor antagonist, has potential therapeutic benefit in inflammatory arthritis by decreasing the concentration of TNFα in rat synovium [162]. It

has also been associated with a decrease VEGF concentration [162] and attenuation of the anti-apoptotic effects of angiotensin II in OA [164].

Cannabinoids

Cannabinoids are thought to be important mediators of anti-nociception as activation of CB2, has been associated with a decrease in nociceptive fiber activity, inhibition of windup pain [217] and a decrease pain scores [218]. In chronic arthritis of rats, local administration of a CB1 agonist resulted in a decreased nociceptive fiber firing rate in response to a mechanical stimulus [167].

Adverse Effects of Intra-articular Receptor Modulation

Diarthrodial joints are well suited to intra-articular injection and the local delivery of therapeutics. Certainly, there are many potential benefits including increased bioavailability, reduced systemic exposure, and decreased adverse events with drugs delivered by this method [219]. However, this method of drug administration is not completely benign. Drugs such as local anesthetics are classically known for their chondrotoxic effects [220,221]. Additionally, the glucocorticoids, particularly triamcinolone, have decreased chondrocyte viability [220,222], while morphine may provide a less chondrotoxic option for intra-articular analgesia [223]. The research on the cartilaginous effects of many intra-articular therapeutics is poorly understood and must be considered prior to administration of these medications. Post-injection flare, as a result of synovitis in response to injected substances, occurs in about 2-6% [224]. Infection, a rare but serious complication of arthrocentesis and intra-articular drug administration, particularly corticosteroids, has been reported in 1 in 3000 to 1 in 50000 patients

[224,225]. In addition to potential adverse effects of intra-articular injection, accurate placement is not always achieved, particularly for joints such as the coxofemoral joint [26]. While intra-articular delivery of therapeutic agents may offer many benefits over systemic administration, the risks associated with intra-articular drug administration must be carefully considered.

A.5 Summary and Conclusion

There are numerous receptors present within the synovium and articular cartilage that regulate many different functions. Based on the literature presented here there are several important receptors in the synovium that modulate nociception such as opioid, glutamate, TRPV, and cannabinoid receptors. The pro-inflammatory effects of TLR, histamine, interleukin, prostacyclin, EGF, and chemokine receptors is balanced by the anti-inflammatory receptors, adenosine, glucocorticoid and cholinergic. Synovial receptors, chemokine, lipoxin, TLR, death, adenosine, and prostaglandin, are important in cartilage maintenance and turnover. As some receptors, opioids, glucocorticoid, glutamate, and interleukin, have important therapeutic implications, further research into local therapeutic modulation of these receptors is crucial.

The activity of synovial membrane receptors represents a balance between cartilage degradation and repair and synovial inflammation and anti-inflammatory effects. Understanding the role of synovial receptors may ultimately help to guide treatment of many arthropathies, particularly OA, by not only modulating cartilage repair and destruction but also by decreasing the pain associated with arthritis.

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Table A.1 Synovial membrane receptors and their actions

Receptor	Receptor Subtype	Proposed Synovial Actions
Opioid		Analgesia [5]
		Anti-inflammatory [11]
Glucocorticoid		Decreased IL-1β [21]
Glutamate		Nociception [26]
Transient Receptor Potential Channels	TRP vanilloid (TRPV)	Increases reactive oxygen species [33]
		Increases COX-2 [34], TNF-α [33,34]
	TRP Melastatin (TRPM)	
	TPRM3	
	TRPM8	
	TRP ankrynin1 (TRPA1)	Hyperalgesia / Cold hypersensitivity [42]
	TRP canonical 5 (TRCP5)	
Chemokine	CCR9	Mononuclear cell infiltration [47]
	CXCR1, CXCR2	Neutrophil chemotaxis [56]
	CCL2	Monocyte adhesion to neutrophils [46]
	CCR9	MMP-3 related cartilage degradation [47]
	CXCR3	Fibroblast invasion [57,58]
Interleukin (IL)	IL-1R	IL-6, IL-8, PGE2, VEGF expression [63]
	IL-22	Synovial proliferation [67]

Receptor	Receptor Subtype	Proposed Synovial Actions
Prostanoid	EP2	Inhibition of IL-1 induced IL-6 release [70]
		Cartilage regeneration [75]
	EP3	Increases IL-6 production [71]
	EP4	Inhibition of IL-1 induced IL-6 release [70]
	Prostacyclin	Inflammatory cell infiltration, Bone and cartilage destruction, IL-1β, IL-6 production [74]
Histamine		Increases IL-1β, IL-6, IL-8 [79], TNF-α release [81]
Bradykinin		IL-1, IL-8 production [81]
		Endothelial cell proliferation [85]
Tachykinin	Neurokinin 1	Unknown
Serotonin		Unknown
Purinergic Receptors	Adenosine	Decreases cartilage destruction [107,108] Decreases IL-8, TNF-α, PGE2 release [104]
	P2	Increases IL-1, IL-6, IL-17, TNF-α [110] Synovial hyperplasia [40]

Receptor	Receptor Subtype	Proposed Synovial Actions
Lipoxin A4		Inhibits neutrophil activation [111,113]
Cholinergic Receptors	α7R	Increases IL-6, IL-8, MMP-3 release [113]
Receptor Tyrosine Kinases	Epidermal Growth Factor	Increases VEGF, IL-8, IL-6 [120] Decreases arthritis score [121]
	Platelet-Derived Growth Factor	Promotes extracellular matrix degradation [126]
	Vascular Endothelial Growth Factor	Promotes OA progression and synovitis; Increases vascular density [128]
	Fibroblast Growth Factor	
	FGFR1	Catabolic effects on cartilage [134]
	FGFR2	Anabolic effects on cartilage [134]
	FGFR3	Catabolic effects on cartilage [134]
	FGFR4	Anabolic effects on cartilage [134]
	Discoidin Domain Receptor 2	Cartilage degradation [136]
Toll-Like Receptors	TLR2	IL-1β, IL-6, MMP-3, TNF-α release [142,144]
	TLR3	IL-6,MMP-3 release [142]
	TLR4	IL-1β, IL-6, MMP-3, TNF-α release [142, 144]
	TLR5	IL-6,MMP-3 release [142]
	TLR7	Cartilage degradation [141] Increases IL-6 and IL-1β [141]
Bone Morphogenetic Protein	BMPR-1A	Cartilage degradation [145,147]

Receptor	Receptor Subtype	Proposed Synovial Actions
Adipocytokine	Adiponectin	IL-6 [148,149], IL-8 [151], PGE2 [152]production
Lipoprotein		IL-6, IL-8 release [155]
Lysophosphatidic acid		Up-regulation of adhesion molecules [158]
Endothelial Protein C		Unknown
Angiotensin II		Unknown
Cannabinoid		Analgesia [167]
Estrogen		Decreases cartilage thickness [174] Decreases cartilage extracellular matrix [180]
Notch		Increases insulin-like growth factor 2, IL-8 expression [180]
Relaxin		Unknown
Death		Cartilage degradation [183]

Table A.2 Local Therapeutic Modulation

Receptor Class	Agonist or Drug	Effect of Intra- Articular Administration
Opioid	Endorphin	Decreased extravasation of leukocytes and plasma proteins [184]
	Morphine in horses	Decreased synovial amyloid A, plasma white blood cell count [185]
	δ- agonist	Decreased bradykinin induced plasma extravasation [186]
	κ- agonist	Decreased myeloperoxidase activity [187]
		Decreased plasma extravasation [186]
Glucocorticoid	Many glucocorticoid agonists	Decrease pain, joint swelling, synovitis [191,197,198] Chondrocyte apoptosis [201]
	Methylprednisone	Decreased glycosaminoglycan synthesis [199]
Glutamate	Ionotropic glutamate receptor antagonist	Reduction in pain [203]
	Metabotropic glutamate receptor antagonist	Reduction in pain [205]
	AMPA and Kainate antagonist	Decreased IL-6 [30]

Transient Receptor Potential Vanilloid	TRPV1 antagonist TRPV4 agonist	Reduction in pain [33,206] Increased plasma extravasation [207] Decreased the pain threshold [207]
Interleukin - 1	Antagonism in bovine cartilage	Decreased glycosaminoglycan release [209]
	Anakinra (antagonist)	Decreased cartilage degeneration Increased lubricin [210]
Tachykinin	Neurokinin 1 antagonist	Decreased cartilage degeneration [92] Decreased mechanical [92] and thermal hyperalgesia [212] Decreased myeloperoxidase activity [213]
Serotonin	Ketanserin (antagonist)	Decreased spinal excitation [215]
	Methiothepin (antagonist)	Decreased spinal excitation [214]
Toll-Like Receptors	Lubricin, inhibits TLR 2,4,5	Decreased synovitis [139] Reduction in pain [139]
Cannabinoid	CB1 agonist	Decreased spinal excitation [167]

Appendix B Radiographic scoring system

Each Determinant to Be Assigned a Value: 0 = Absent, 1 =Mild, 2 = Moderate, 3 = Severe	Score
Patellar osteophytes	
Femoral trochlear groove periarticular osteophytes	
Lateral and/or medial condylar periarticular osteophyte	
Femoral subchondral sclerosis	
Distal femoral condylar remodeling	
Subchondral cystic lucencies	
Sesamoid periarticular osteophytes	
Femoral intercondylar notch osteophytes	
Proximal tibial periarticular osteophytes	
Proximal tibial subchondral sclerosis	
Proximal tibial subchondral cystic lesions	
Central tibial plateau osteophytes	
Joint effusion/capsular thickening	
Intraarticular mineralized osseous fragments	
Meniscal mineralization	
Intercondylar avulsion fracture fragments	
	Total

Appendix C Diet questionnaire completed by all caregivers of canine patients enrolled in the clinical trial.

1. What diet is your dog currently consuming (please list specific diet including whether dry only, canned only or both dry and canned).

2. How often are you feeding your dog each day?

3. What quantity of food is fed at each feeding?

4. How long has your dog been on this diet?

5. Does your dog receive treats?

a. If so, how often does your dog receive treats?

b. How many treats does your dog receive each day

6. Is your dog receiving any supplements? Supplements include but are not limited to fish oils, omega – 3 fatty acids, glucosamine, chondroitin, Dasuquin, Cosequin, etc.

a. If so, what supplements is your dog receiving?

7. Is your dog currently taking any medications other than flea, tick or heartworm preventatives?

a. If so, what medication(s) is your dog receiving?