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THE EFFECTS OF GENE THERAPY IN AN OVINE OSTEOARTHRITIS MODEL

by

Crystal Collier

A thesis submitted in partial fulfillment  
of the requirements for the degree

of

MASTER OF SCIENCE

in

Animal, Dairy, and Veterinary Sciences

Approved:

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Jeffrey B. Mason, Ph.D.  
Major Professor

---

S. Clay Isom, Ph.D.  
Committee Member

---

Arnaud Van Wettere, Ph.D.  
Committee Member

---

Richard S. Inouye, Ph.D.  
Vice Provost for Graduate Studies

UTAH STATE UNIVERSITY  
Logan, Utah

2020

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## ABSTRACT

## The Effects of Gene Therapy in an Ovine Osteoarthritis Model

by

Crystal Collier, Master of Science

Utah State University, 2020

Major Professor: Jeffery B. Mason, Ph.D.  
Department: Animal, Dairy and Veterinary Sciences

Osteoarthritis (OA) is the most common joint disorder in the U.S. It becomes most prevalent in adults 60+ years of age and causes decreased mobility, discomfort, and in some cases, excruciating pain. The ovine osteoarthritis model is effective in advancing the understanding of the disease. In osteoarthritis, aggrecanase enzymes are upregulated and contribute to the degradation of one of the key proteoglycans of cartilage, aggrecan. Tissue Inhibitor of Metalloproteinases (TIMPs) inhibit aggrecanases, which maintains the enzyme activity in balance. The current study focused on the up-regulation of TIMP-3 protein *in vivo* using viral delivery of a *TIMP-3* transgene directly into the stifle joint of sheep. It was hypothesized that *in vivo* up-regulation of cell-produced, TIMP-3 protein would decrease proteoglycan degradation and slow OA progression. We surgically induced OA in an ovine model using an induced-trauma or hormone-depletion approach, combined with oblique-angle forced exercise to accelerate OA progression. Mature ewes underwent ovariectomy ( $n = 7$ ) or cranial cruciate ligament desmotomy ( $n = 8$ ) and were

exercised at an oblique angle for four months to accelerate induction of osteoarthritis. The *TIMP-3* treatment numerically decreased gait stance time, average osteophytosis score, and joint widening, but did not significantly affect joint space narrowing. The injected *TIMP-3* transgene also numerically decreased aggrecanase activity and serum glycosaminoglycan content and increased urine glycosaminoglycan content. This study demonstrated the positive potential of *in vivo* gene therapy for the treatment of OA.

(116 pages)

## PUBLIC ABSTRACT

## The Effects of Gene Therapy in an Ovine Osteoarthritis Model

Crystal Collier

Humans rely on the health of their joints for stability and mobility in daily life. In a normal, healthy functioning joint, complex processes maintain joint tissues, including remodeling, lubrication, and immune function among many other tasks. Osteoarthritis (OA) is the most common joint disorder in the U.S. It becomes most prevalent in adults 60+ years of age and causes decreased mobility, discomfort, and in some cases, excruciating pain. In the biological processes of osteoarthritis, the metalloproteinase enzymes responsible for the degeneration of cartilage are upregulated. The inhibitors of metalloproteinase activity are known as Tissue Inhibitor of Metalloproteinases (TIMPs), which keep metalloproteinase activity in balance. However, an increased production of metalloproteinases has been noted in osteoarthritis-influenced cartilage. The current study focused on the up-regulation of TIMP-3 protein *in vivo* using the introduction of a *TIMP-3* transgene. It was hypothesized that up-regulation of cell-produced, TIMP-3 protein would decrease proteoglycan degradation and slow OA progression *in vivo*. The use of sheep as a model has proven an effective technique for characterizing diseased joint issues and with our demonstrated method of inducing osteoarthritis over a period of months instead of years, we can make strides in advancing the understanding of the disease. Our methods involve two groups of surgically altered, mature, female sheep. One half underwent ovariectomy or removal of both ovaries ( $n = 7$ ) and the other half, a

Cranial Cruciate Ligament Desmotomy or transection of the cranial cruciate ligament, similar to the anterior cruciate ligament in humans ( $n = 8$ ). OA was observed in sheep with these surgeries that were subjected to forced exercised at an oblique angle for four months. We found that the *TIMP-3* treatment decreased gait stance time, average osteophytosis score and joint widening, but did not significantly affect joint space narrowing. The *TIMP-3* transgene also decreased aggrecanase activity and serum glycosaminoglycan content and had a positive effect on urine glycosaminoglycan content. Through understanding and utilizing these new methods, we hope to refine our model to further research in this field and offer a better solution for those affected by OA.

## ACKNOWLEDGMENTS

I am overwhelmed with gratitude towards all of those who assisted me in my endeavor to complete this research project and earn this degree. I would first like to acknowledge my close family and friends, especially my spouse, Gareth, for their relentless support through thick and thin. Gareth helped me tackle and train sheep and stayed late with me or waited long hours as I worked in the lab. He had the patience to plan a wedding with me in the middle of the summer sheep runs in 2018 and support me in that simultaneously. He stuck by me in the overwhelmed late nights and busy, early mornings. I recall a specific few days while we were dating, and I was very ill. He drove me to the Poisonous Plants Research Lab where some sheep were recovering from surgery, caught each one as I looked on shivering, tackled each to the ground (usually a two-person task) so that I was able to collect a heart rate, breathing rate, temperature and assess the surgery sites and drove me home to my sick bed. He even contributed intellectually to some of my writing all the while calling me his smart “science-y wife.” My family and friends were very supportive as well, having patience during my daily, time-consuming sheep runs and never-ending lab tasks. I appreciate them for taking interest in what I was doing and the praise and encouragement I received from them.

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surgeries over the course of just 3 days. I want to thank my lab mates, especially Peter Welsh, Anisa Samhouri, Kate Parkinson, and Tracy Habermehl, for their help in surgery, recovery, the sheep runs, collections, and seemingly endless lab work and assays. Thank you to Peter, for offering great advice and practical insights. I am grateful to Anisa for her invaluable work ethic and intellectual support. Kate kept me organized and was always encouraging and helpful in the lab and in the field. Thank you to Tracy for help with everything related to the sheep from training to running, surgery to collection, assays to writing she was there for it all. These and many others were willing to get their hands dirty right alongside me tackling 150+ lb. animals, collecting bodily fluids and pipetting until their fingers cramped.

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Crystal Collier

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## LIST OF ABBREVIATIONS

AAV	= Adeno-Associated Virus
ACL	= Anterior Cruciate Ligament
ACLT	= Anterior Cruciate Ligament Transection
ADAM	= A Disintegrin and a Metalloproteinase Domain
ADAMT	= A Disintegrin and Metalloproteinase with Thrombospondin Motifs
AdV	= Adenovirus
AS	= Ankylosing Spondylitis
BMI	= Body Mass Index
BMPs	= Bone Morphogenetic Proteins
CaCL	= Caudal Cruciate Ligament
CCLD	= Cranial Cruciate Ligament Desmotomy
CrCL	= Cranial Cruciate Ligament
CS846	= Chondroitin Sulphate Epitope 846
DEG	= Differentially Expressed Gene
DHEA	= Dehydroepiandrotestosterone
DHEA-S	= DHEA Sulfate
DHT	= Dihydrotestosterone
DKK-1	= Dickkopf-1
ECM	= Extra Cellular Matrix
ELISA	= Enzyme Linked Immunosorbent Assay
FRP	= Frizzled-Related Protein
GAGs	= Glycosaminoglycans
GRF	= Ground Reaction Force
HA	= Hyaluronic Acid
LV	= Lentivirus
MCL	= Medial Collateral Ligament
MCLT	= Medial Collateral Ligament Transection
Micro-CT	= Microcomputed Tomography

MMP = Matrix Metalloproteinase

MRI = Magnetic Resonance Imaging

MSC = Mesenchymal Stromal Cell

OA = Osteoarthritis

OARSI = Osteoarthritis Research Society International

OP = Osteoporosis

OVX = Ovariectomy

PPRL = Poisonous Plants Research Laboratory

PTH = Parathyroid Hormone

RA = Rheumatoid Arthritis

rAAV = Recombinant Adeno-Associated Virus

TACE = TNF- $\alpha$  converting enzyme

TIMPs = Tissue Inhibitor of Metalloproteinases

UVDL = Utah Veterinary Diagnostic Laboratory

WNT = Wingless-Related Integration Site

WOMAC = Western Ontario and McMaster Universities Osteoarthritis Index

## **CHAPTER I**

### **INTRODUCTION**

The normal and healthy function of joints is essential for pain-free daily life. Synovial joints are comprised of two opposing/articulating surfaces. The major components include bone, cartilage, synovium and the joint capsule. These tissues have specialized cells, proteins, and pathways that regulate the remodeling of bones and joints.

The synovial joints are also the joints that are most commonly susceptible to osteoarthritis (OA). Millions of people suffer from OA, with the majority being women over the age of 60. Osteoarthritis is a complex disease with various symptoms including mild to severe pain, loss in the range of motion, and joint stiffness (Z. Li et al., 2019). In most osteoarthritic joints, an imbalance develops in the normal remodeling homeostasis within the joint tissues, including articular cartilage. Increased activity in catabolic enzymes specializing in the degradation of particular components of cartilage often cause a thinning of cartilage and can include a decrease in joint space. This thinning of cartilage puts more pressure on the subchondral bone below, spurring the formation of osteophytes and bone sclerosis. The general mechanisms behind the formation of osteophytes are an escalation in the formation of new bone and a decrease in bone resorption. Osteoarthritis can be classified as either primary or secondary OA (Saxon, Finch, &, 1999). Primary OA occurs without an obvious causative event, that is, from aging or 'wear-and-tear'. Secondary OA occurs from an underlying condition or causative event such as injury. Obesity, excessive exercise/overuse and genetics can also play a role in the development and progression of the disease (Mandl, 2019).



Treatment of OA falls into three main categories: nonpharmacological, pharmacological, and surgical. If a patient is overweight, it is recommended that diet and exercise changes be to decrease weight and relieve stress on joints. Another non-pharmacological approach is the use of physical therapy to keep the joint moving and alleviate pain. Pharmacological methods include viscosupplementation or treatments injected into the synovial fluid to decrease the breakdown of cartilage, non-steroidal anti-inflammatory drugs, and opioids for pain relief. When OA has progressed too far for an acceptable quality of life, there is the option of surgery through arthroscopy or partial or total joint replacement. Arthroscopy is a non-invasive, exploratory surgery where a surgeon can examine the state of the joint more thoroughly and perform minor fixes such as the removal of loose cartilage, debridement, washing of the joint, and suturing. A patient can also have a portion, or all of the affected joint removed and replaced often resulting in a return to relatively normal joint function (Arif, Ahmad, & Arif, 2019).

Many animal models have been used in the study of OA to facilitate more in-depth research than can be done with humans. Common animal models include rodents, rabbits, dogs, sheep and goats, pigs and horses (Teeple, Jay, Elsaid, & Fleming, 2013). The dog has often been used because of the frequency of arthritic and joint-injured dogs being seen by veterinarians. There are several canine anterior cruciate ligament transection models, but researchers are moving away from companion animal models, as smaller livestock animals are more readily available with less social controversy and similar benefits. Sheep are easy to handle and have joints anatomically similar to humans. The equine model is popular because the equine industry is a large and often lucrative

target industry. There are many orthopedic injuries and diseases in horses, including OA, which if effectively treated, could have a great economic return due to the horse's many uses in sports, work, and leisure.

To model secondary OA, trauma to the joint or other connective tissues is a reliable method for initiating OA. Common methods of trauma induction include the transection of major ligaments, excessive or repetitive loading of joints or the direct damaging of cartilage through mechanical or chemical injury. Another method for OA induction is the manipulation of hormonal influence on joint tissues. When a woman enters the menopausal transition, her probability of developing OA increases significantly (Prieto-Alhambra et al., 2014). Ovariectomy is used to induce these hormonal changes as a 'menopausal' mode of OA induction. Forced exercise can accelerate the development of OA due to changes in pressure and impact angle and can increase the damage to joints already compromised.

The degradation of connective tissues, particularly cartilage, plays a large role in the pathogenesis of OA. It is within cartilage's extracellular matrix (ECM) that out-of-balance remodeling enzymes attack critical proteins and break down critical matrix architecture. One of the most important proteins degraded is aggrecan, a large proteoglycan that makes up an extensive percentage of the cartilage matrix and displays critical water-holding capacity (Fox, Bedi, & Rodeo, 2009). Aggrecan is often used as a biomarker for the early onset of OA because most clinical signs of the disease do not appear until later stages. Another biomarker is total glycosaminoglycan (GAG) content detected either in the serum, urine or remaining in the cartilage tissue.

One of the most common clinical biomarkers of OA is osteophytosis or bone spurs. This is an overgrowth of bone that appears as small outgrowths on the edges of the articular surface and commonly appears in moderate to severe OA. Additional skeletal changes often include a decrease of joint space due to cartilage thinning, an increase in joint width accompanying osteophytosis, osteopenia or the thinning of bone and sclerosis or the hardening of bone. Measurements of gait have also been used to assess pain and joint function in patients with OA. A decrease in speed of gait, stride length, maximum joint angle, and stance time are all evaluations of gait that can be used in disease diagnosis (Neogi, 2012).

Within the complex environment of the joint, several genes regulate various signaling pathways and actions that play a role in the development of OA. The Wnt signaling pathway is a main driver in the development, repair, and maintenance of joint tissues (Amrein et al., 2014). One of its inhibitors, Dkk-1, has been shown to help keep Wnt signaling and bone remodeling in balance (Pinzone et al., 2009). Sclerostin is another remodeling protein produced by specialized bone cells called osteocytes, which also inhibits the Wnt pathway (Bonewald & Johnson, 2008).

Gene therapy is a comparatively new treatment for genetic deficiencies. The main concept is to deliver a specific gene to an organ or specific tissue in the body where the gene product is deficient or faulty (Cross, 2019). The transgene can up/down-regulate the production of a gene product and return the altered function back to normal. Some possible candidates for gene therapy in OA are the tissue inhibitors of metalloproteinase (TIMPs), as they block enzymes that degrade specific proteins within the ECM. An

especially good candidate for this is *TIMP-3* because the TIMP-3 protein binds firmly to the ECM and can inhibit specific metalloproteinases (MMPs) that regulate bone development (Gooz, 2010). Tissue inhibitor of metalloproteinase-3 also prevents the activation of (tumor necrosis factor-alpha) TNF- $\alpha$  by inactivation of the tumor necrosis factor-alpha converting enzyme (TACE or ADAM17). This reduces inflammatory pathway activation, a common occurrence in OA. A problematic aspect of gene therapy can be in determining an effective vector for the transport of the transgene into the target cells. Many viral vectors have proven effective in this pursuit due to their excellent ability to infect cells and integrate their DNA. An adeno-associated virus (AAV) is a notably safe vector as it is non-integrating (most often remains episomal) with a decreased chance of insertional mutagenesis and is not associated with any known pathologies (Tomar, Matta, & Chaudhary, 2003).

The current study aimed to develop a gene therapy approach utilizing viral vectors to regulate the development/progression of OA. We used surgical induction of OA in an ovine model with an induced-trauma or hormone-depletion approach, combined with oblique-angle forced exercise to exacerbate OA progression. The efficacy of the recombinant adeno-associated viral vector (rAAV) delivery of a *TIMP-3* transgene into the stifle joint of a sheep to influence the development/progression of OA was tested.

## **CHAPTER II**

### **REVIEW OF LITERATURE**

#### **Normal Joint Function**

Humans rely on the health of their joints for stability and mobility in daily life. In a normal, healthy functioning joint, complex processes maintain joint tissues, including remodeling, lubrication, and immune function among many other tasks. The main tissues involved in articular joint function are cartilage, bone, synovium and surrounding joint capsule (Lozada & Diamond, 2020). Each type of tissue is highly specialized to ensure proper control, smooth movement and regulated interaction among tissue types. There are three types of joints within the body: fibrous (immovable), cartilaginous (partially moveable) and synovial (freely moveable). A synovial joint's purpose is to facilitate motion using the articulating surfaces of lubricated articular cartilage. These joints often contain a stabilizing meniscus tissue structure as well.

#### **Articular Cartilage**

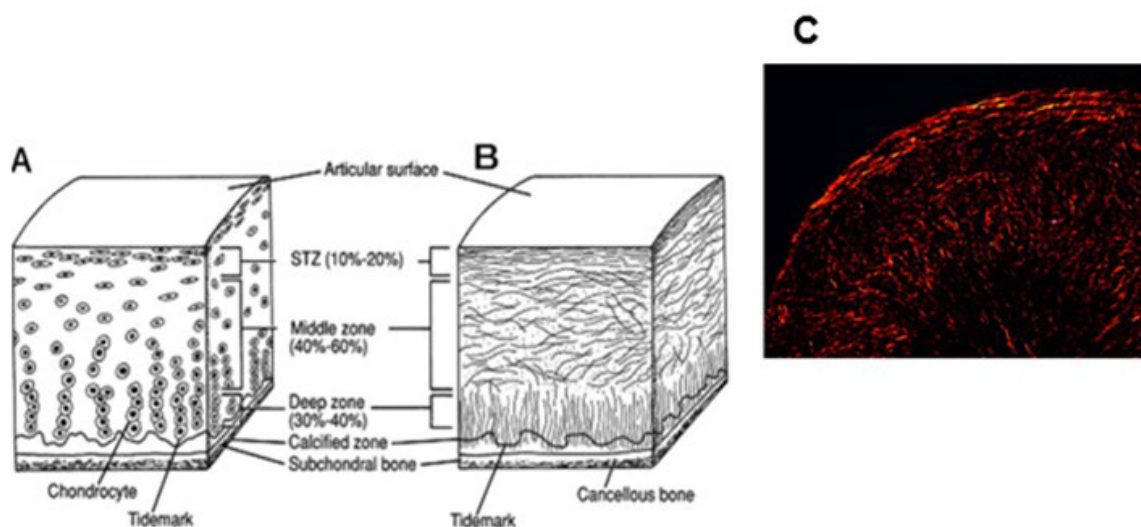
The main function of cartilage within a joint is to act as a smooth, lubricated, articulating surface that can absorb the impact of regular joint movements (Fox et al., 2009). The cartilage acts to distribute impact/weight over a wide surface to reduce joint trauma while a person walks, runs, jumps, and dances. It has shock-absorbing and friction-reducing functions (Lozada & Diamond, 2020). Cartilage tissue lacks direct blood flow but is supplied with nutrients and disposes of wastes through the synovial fluid and to a lesser degree, the underlying subchondral bone. The lack of direct blood

supply reduces the healing capacity of the articular cartilage. To maintain the health of cartilage, its complex architecture needs to be considered.

There are three main types of cartilage in the body: hyaline, fibrous and elastic. Of these, hyaline cartilage is the most common and is found in the ribs, nose, trachea, larynx and many surfaces of bones within joints. Hyaline cartilage provides a smooth surface on which joints can glide. It is meant to reduce pressure on the underlying subchondral bone (Bhosale & Richardson, 2008). Within the hyaline group is a subgroup known as articular cartilage, which is located within articular joints (Fox et al., 2009). Articular cartilage is comprised of chondrocytes and an extracellular matrix (ECM). The ECM contains water, collagen, proteoglycans, and other non-collagenous proteins and glycoproteins (Lozada & Diamond, 2012). Water makes up between 65% and 80% of the ECM in articular cartilage (Fox et al., 2009; Griffin & Guilak, 2005). Chondrocytes are specialized cells that help to rebuild/remodel the ECM (Tamer, 2013).

Articular cartilage can be typically be divided into four zones: the superficial zone, middle zone, deep zone, and calcified zone (see Figure 1). The superficial zone is the first line of defense against stress to the cartilage. This zone is ductile but tough, with collagen fibers aligned parallel to the articulating surface. The middle or transitional zone then provides more resistance to impact. It contains less water and more proteoglycans, with collagen fibers aligned perpendicular to the articulating surface. The deep zone of articular cartilage is the most compact and has the highest proteoglycan content and lowest water content. Its purpose is to protect the subchondral bone from the heaviest forces. Finally, the calcified zone attaches the cartilage to the bone beneath. There are not

many cells within this zone. Collagen fibrils secure themselves to the subchondral bone (Fox et al., 2009).



*Figure 1.* The cellular population and fibril structure of articular cartilage throughout the zones. A depiction of the cellular population and collagen orientation of articular cartilage throughout the zones (A). A depiction of the collagen and fibril structure of articular cartilage throughout the zones (B, Fox et al., 2009; C, unpublished data).

These zones can be further subdivided into three regions: the pericellular region, territorial region, and interterritorial region. These regions are specifically distinguished by the way the ECM is structured (different structures in specific regions), the position of the ECM relevant to chondrocytes, and the organization. Surrounding the chondrocyte, the pericellular matrix consists of mostly proteoglycans. It also contains glycoproteins and non-collagenous proteins. The pericellular matrix (lacunae) is enveloped by the territorial matrix. Much of the composition of the territorial matrix is collagen fibrils that protect the chondrocytes and other cartilage cells from excess stress. Finally, the interterritorial matrix is the main contributor to the biomechanical properties of articular cartilage. This region is the largest and has a generous proteoglycan content (Fox et al.,

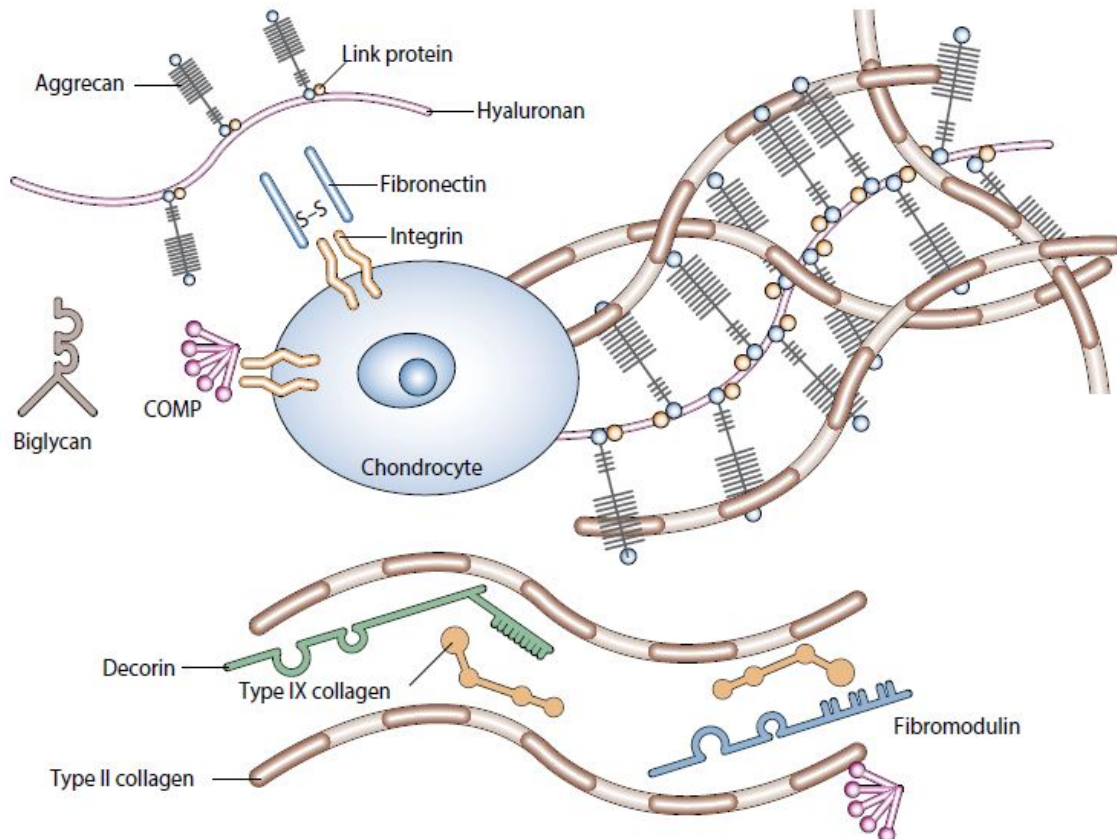
2009).

Chondrocytes are the main cells of articular cartilage. They act as maintenance cells for the ECM. Chondrocytes are rarely involved in intercellular interactions and predominantly rely on various stimuli within their personalized microenvironment within the ECM. The chondrocyte must reside in a very specific environment to function properly and synthesize most of what is needed to maintain homeostasis within the ECM (Bhosale & Richardson, 2008). Unfortunately, chondrocytes encapsulated within the dense ECM have limited capacity for functional replication, which contributes to the limited ability for cartilage to heal (Luria & Chu, 2014).

Glycoproteins are a major component of the articular cartilage matrix. Cartilaginous collagens occur in multiple types. Of these types, type II collagen is the major form present (see Figure 2). Other collagens present include type I, III, IV, V, VI, IX, X and XI. Their role is to provide supportive links/crosslinks, assist in type II collagen formation/ orientation and help sustain the network of fibrils and fibers. Proteoglycans are another type of protein present in articular cartilage. After collagen, they are the largest group. Proteoglycans are essential for the normal function of cartilage. Proteoglycans use their inherent osmotic properties to increase the capacity of articular joints to absorb intense loading. They also assist in the management of fluid and electrolyte balance within the matrix. One of the largest proteoglycans is aggrecan. There are also decorin, fibromodulin, and biglycan. These proteins are produced by chondrocytes and secreted into the ECM. Aggrecan's structure consists of chondroitin sulfate and keratan sulfate chains and can interact with hyaluronan and create large



proteoglycan aggregates. Chondroitin sulfate and keratin sulfate are two major types of GAGs, which are subunits of proteoglycans. A breakdown and loss of aggrecan have been repeatedly identified in the degradation and destruction of cartilage (Bhosale & Richardson, 2008; Fox et al., 2009).



*Figure 2.* The main structure and components of the extracellular matrix of articular cartilage. Illustrated here are the two main proteins within the matrix: collagens and proteoglycans. (Tamer, 2013)

### Subchondral Bone

Each articular joint involves the articulation of at least two separate bones. The cartilage and synovium are engineered to protect these articulating bones from stress, strain, and abrasion. Bone is chiefly made up of type I collagen proteins contributing to a

dense ECM (Javaheri et al., 2016). Key cellular players in the structure and remodeling of bone include osteoclasts, which degrade bone, osteoblasts, which synthesize bone and osteocytes, which are bone cells embedded in the matrix that signal osteoclasts and osteoblasts.

Subchondral bone refers to the part of the bone that is just beneath the cartilage. Subchondral bone can be further subdivided into two separate categories: the subchondral bone plate and the subchondral trabecular bone. The subchondral bone plate is very porous and provides a network for the calcified cartilage layer and subchondral trabecular bone to interact. The subchondral bone plate allows blood and nerves to travel through it. Subchondral trabecular bone is even more porous than the subchondral bone plate. It contains not only blood vessels and nerves but also the bone marrow. Overall, subchondral bone has a dynamic design and is specialized for the resistance of frequent and harsh forces taken on by synovial joints. This stress also has the potential to change the structure of subchondral bone, as it can adapt through bone modeling and remodeling (G. Li et al., 2013). Trabecular bone in the female is subject to greater remodeling regulation than in males due to the potential mineral demands during fetal development and lactation.

Modeling and remodeling within the subchondral bone is a task completed by the intricate collaboration of osteoclasts, osteoblasts and osteocytes. Osteoblasts, the tissue builders, cannot work alone to synthesize bone tissue. They work in groups of many connected cells. In bone remodeling, there is a coordinated effort between osteoblasts, and osteoclasts to discard biomechanically inferior portions of bone and replace it with

new bone. When either the osteoblasts or osteoclasts are delayed in their endeavors, osteoarthritis or osteoporosis can occur. In addition to these two types of bone remodelers, a third specialized cell is the osteocyte. An osteocyte is formed when an osteoblast implants into the matrix it has deposited. Osteocytes do not divide but can survive up to 25 years within the human body. Osteocytes act as signalers for osteoblasts and osteoclasts telling them when and where to remodel damaged or inferior bone (Stewart & Kawcak, 2018).

In addition to the three main cell types in bone structure, there are many other factors in bone organization, assembly, composition, regulation, remodeling, and repair. The mechanisms involve the Metzincin family of enzymes, which include the matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs) enzymes, a disintegrin and a metalloproteinase domain (ADAMs) enzymes, growth factors, other cytokines, and their receptors, and the TIMPs (Javaheri et al., 2016).

### **Synovium and Joint Capsule**

Synovial joints are those that freely move (Tamer, 2013). The joint capsules typically enclose the whole of the synovial joint, provide a sterile joint environment, and are very important in keeping the synovial joint in proper position while the joint is in motion (Ralphs & Benjamin, 1994). The internal surface is lined with the synovial membrane (Tamer, 2013). Within the synovial membranes are highly specialized cells known as synoviocytes. Synoviocytes are very important in the production of synovial fluid and all its components. They produce synovial fluid through the ultrafiltration of

blood plasma. They also make hyaluronic acid (HA), which is a major, non-cellular component of the synovial fluid. Because articular cartilage is avascular, it relies on the synovium to deliver nutrients and clear wastes (Lozada & Diamond, 2020).

A major function of synovial fluid is to lubricate the joint and to serve as a biochemical pool where nutrients and regulatory cytokines can travel (Tamer, 2013). The synovial fluid also contributes to shock absorbance by providing flexibility and viscosity to the joint (Lozada & Diamond, 2020). Synovial fluid contains molecules that help provide a low-friction environment including proteoglycan 4, HA, and surface-active phospholipids. In disease states, the synovium may show significant changes, even before visible cartilage degeneration has occurred, with infiltration of mononuclear cells, thickening of the synovial lining layer and production of inflammatory cytokines.

### **Osteoarthritis**

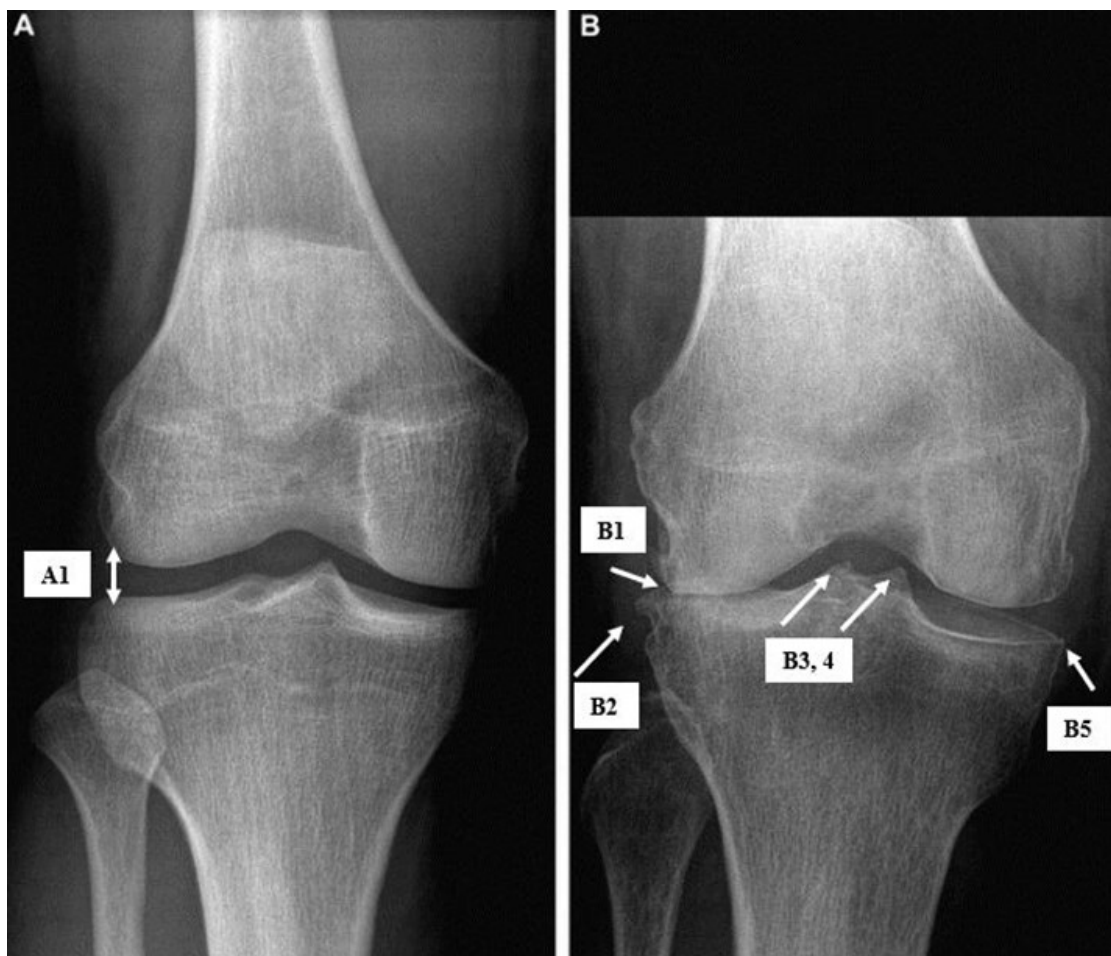
Osteoarthritis is the leading joint disease among humans throughout the world. According to a review by L. A. Mandl (2019), over 22.7 million Americans suffer limitations due to the symptoms of OA. The World Health Organization ranks OA as 1 of the 10 most disabling diseases (Poulet, 2016). Because the world population is aging, stemming from an increase in human longevity, aged populations are expected to increase steadily in the coming years. Although age is considered the leading risk factor for the development of OA, other contributing factors may include obesity, injury, gender-dependent risk factors (including menopause), and genetics. Osteoarthritis is a major cause of debilitation and chronic pain within the U.S. and internationally. Worldwide, it is estimated that knee OA occurs in 3.8% of people and 43.5% of those

afflicted suffer from limitations in physical activity due to pain and discomfort (Z. Li et al., 2019). In a study completed by Wallace et al. (2017), it was determined that the modern, cultural shift toward a decrease in overall physical activity has had a significant impact on muscles, making them weaker and, therefore, less able to support and control joint movement. This can lead to a greater susceptibility to OA in the present day, compared to the average person 100 or more years ago.

Osteoarthritis can be defined as the breakdown of joint tissues and inflammation within the joint. This can occur over time with constant joint loading, with an increase in the overall Body Mass Index (BMI) or weight of an individual (adding additional stress to joints) and through injury, which may disrupt the normal joint environment (Wallace et al., 2017). A decrease in overall physical activity in the mid to late 20<sup>th</sup>-century population has resulted in under-loaded joints with decreased proteoglycan content and weaker muscles. These factors reduce joint stability and lead to greater wear and an increased risk of injury (Mandl, 2019).

An osteoarthritic joint differs from a normal healthy joint in that within the diseased joint, the normal remodeling process is disrupted providing an environment for excessive cartilage breakdown, excess bone growth/degradation, and inflammation. The articular cartilage, in particular, experiences significant disruption. Osteoarthritis affects every aspect of the joint, including cartilage, synovium and subchondral bone (Z. Li et al., 2019; Roemhildt et al., 2010). Osteoarthritis is most commonly found in weight-bearing joints, including knees, hips, spine, fingers, and feet (Lozada & Diamond, 2020). A clinical hallmark of OA is osteophyte growth. Osteophytes are sections of bone growth

that can occur coincident with the breakdown of cartilage. Sclerosis or bone hardening in the subchondral bone can occur as well as (and is often preceded by) osteopenia or bone thinning, synovitis or inflammation of the synovium and capsular fibrosis, which can interfere with the normal functional architecture and metabolism of the joint (Aziz, Iqbal, Aziz, Shafaat, & Jilani, 2019). Cartilage breakdown often results in pain on impact and a decrease in joint space that can be viewed radiographically (see Figure 3). Along with cartilage breakdown, bone incurs lesions and erosion as OA progresses (Lacourt et al., 2012).



*Figure 3.* A caudal-cranial radiograph of the femorotibial joint without (A) and with OA (B) revealing migration of the femur and tibia closer together (A1 to B1). There is also visible osteophytosis (B2-B5) (Altman & Gold, 2007).

Osteoarthritis is normally slowly progressive over time, but it is also a very active process (Arif et al., 2019). Age is a major risk factor for OA and younger patients normally heal better than older subjects (Bhosale & Richardson, 2008; Griffin & Guilak, 2005). With increasing age, hydration of the ECM is decreased, and the symptoms of OA can be more severe (Fox et al., 2009).

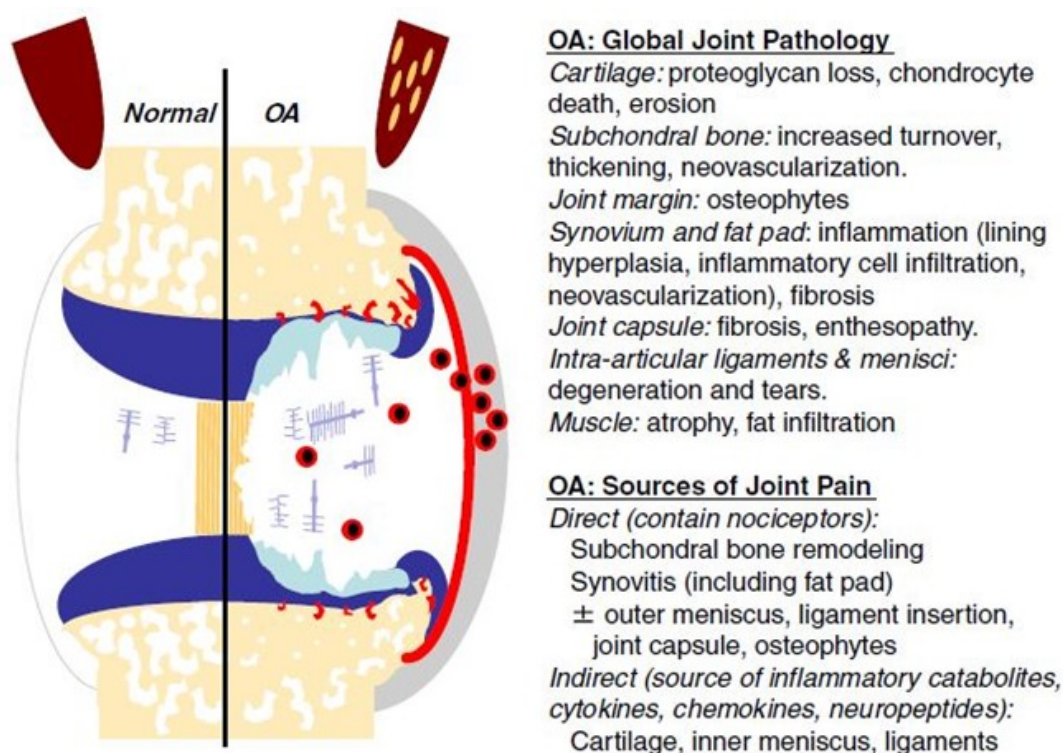
The most commonly described symptoms of osteoarthritis include pain (mild discomfort to severely debilitating), a loss in range of motion, stiffness, and inflammation (Z. Li et al., 2019). Joint stiffness often occurs after a patient has been at rest for extended periods, including overnight (Lozada & Diamond, 2020). Aching joint pain is common and often exacerbated by overuse. The underlying mechanisms are still being studied.

In a study published by Chen et al. (2019) at the Wan Fang College of Medicine in Taiwan, it was determined that both severe and stable joint pain is associated with anxiety and depression, especially in older patients. As OA commonly affects middle-aged to older people, these psychological impairments are often associated with physical limitations and pain (Chen et al., 2019). Of all the symptoms of OA, it has been reported that impairment of function is the most distressing to patients. Impairment of function is manifest in various ways, such as a limit on the distance a patient can walk, limping, difficulty using stairs and difficulty performing normal daily activities. Another symptom of OA is bony crepitus (the grinding of joints) and sarcopenia due to OA-associated impairment or disability. Patients suffering from OA often experience acute flares, as well as periods of remission (Aziz et al., 2019).

OA is often classified as non-inflammatory arthritis. However, there is increasing



evidence of inflammatory involvement (Lozada & Diamond, 2020). In an osteoarthritic environment, there is often increased cytokine production, cellular infiltration, and inflammatory activation in articular tissues (Lieberthal, Sambamurthy, & Scanzello, 2015). Inflammatory cytokines are critical regulators in the production of enzymes that can disrupt cartilage (Figure 4). In a study published by Bigoni et al. (2012), the concentration of various interleukins was recorded post-anterior cruciate ligament (ACL) injury in male human patients. Data were gathered from synovial samples collected at different time points and demonstrated a significant increase in specific groups of anti-inflammatory molecules.



*Figure 4.* A depiction of the complex, multi-faceted, dynamic pathology of osteoarthritis. The pathology of the disease has been studied, but the exact mechanisms have not been determined (Malfait & Little, 2015).



Z. Li et al. (2019) reported that OA is polygenic. The process of thinning cartilage is hypothesized to stem from an imbalance in remodeling enzymes, specifically MMP enzymes and TIMP enzymes. In the above-mentioned study (Z. Li et al., 2019), a dataset including 22 knee OA patients and 22 control patients was used to discover differentially expressed genes (DEGs), where 229 genes were identified as differentially expressed. The top 10 up-regulated genes functioned in inflammatory and immune pathways, chemokine activity and cytokine activity (Z. Li et al., 2019). Although many genes participate in the pathology of OA, some have been determined to have a particular impact on the progression of the disease. In a study published by Karlsson et al. (2010), 11 genes displayed increased expression in osteoarthritic cartilage, compared with healthy cartilage. These genes (CLEC3B, CDH11, GPNMB, CLEC3A, CHST11, MSX1, MSX2, COL13A1, COL14A1, COL15A1, and COL8A2) are involved in bone and collagen formation (Karlsson et al., 2010).

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by the body's immune system attacking its own joints resulting in major inflammation, joint stiffness, pain, and fatigue. Rheumatoid Arthritis contrasts with OA in a few ways. Rheumatoid Arthritis is considered a systemic condition whereas OA is considered to be localized to one or more individual joints (Saxon et al., 1999). Chronic joint inflammation is the most prominent symptom of RA and is hypothesized to be caused by antigen-stimulated T cells infiltrating the synovial membrane. Although inflammation is a prominent symptom in OA, it is temporal in nature and is not the primary defining characteristic, as it is in RA. Other symptoms of RA include tissue destruction in the

joint. Rheumatoid Arthritis destroys cartilage. However, inflammation decreases once the cartilage is destroyed or surgically removed (Førre, Haugen, M., & Hassfeld, 2000).

Because RA is an autoimmune disease, there are different measures in creating an animal model for this disease, whereas OA can be induced surgically, hormonally, and through joint loading.

### **Current Treatments**

Goals for the treatment of OA include the reduction of pain and functional impairment, improvement of joint mobility and reduction in the progression of the disease (Chen et al., 2019). Because OA is such a prevalent and debilitating disease, many and various therapies have been researched and tested to alleviate the pain and disability it can create.

The medical management of OA is currently divided into three general phases. The first is non-pharmacological management such as weight loss through proper diet and exercise and physical therapy. The second is pharmacological management using analgesics such as nonsteroidal anti-inflammatory drugs (NSAIDs), opioid analgesics, viscosupplementation, corticosteroid injections, and intra-articular HA (Arif et al., 2019). The third phase is the use of surgical management such as total joint replacement surgery in the hip or knee (Aziz et al., 2019). All these treatments for OA do not prevent suffering, they only alleviate the pain that has already manifested itself enough to warrant a trip to the doctor (Mastbergen et al., 2006).

Usually, the first step in the prevention and management of OA is a

nonpharmacological approach. The increased stress caused by being overweight is a large factor in the etiology of some patient's disease. Obesity increases the potential for developing OA, especially in the knee (Saxon et al., 1999). Subsequent to the effects of obesity, diabetes, decreased estrogen production and increased high-density lipoprotein cholesterol may also play roles in the development of OA. Compounding on these factors is the positive correlation between body weight and aging, as metabolism and physical activity tend to slow down (Christensen et al., 2017). Aging is a commonly accepted factor in OA progression and the side effects can contribute to a cascade of related morbidities, including obesity. Weight gain and being sedentary after an injury are common contributing factors in the development of OA. In a study on rats that underwent an anterior cruciate ligament transection plus medial collateral ligament transection (ACLT + MCLT), there was significant weight gain noted in injured rats as opposed to controls (Sudirman, Ong, Chang, & Kong, 2018). With the escalating factors of pain due to injury, pain due to OA and even pain due to other age-related diseases, it is difficult for those suffering from OA to address these issues through physical exercise.

In a study by Bliddal, Leeds, and Christensen (2014), change in physical function was measured against weight loss or gain. There was a distinct decrease in the physical function of those individuals who gained weight compared to those who lost or remained the same. A review by Gill et al. (2011) explored the results of many studies involving bariatric surgery as a weight-loss tool and the subsequent improvement of OA. Overall, positive results suggested that bariatric surgery, due to the common weight loss effect, is positively correlated with the improvement of OA.

Another nonpharmacological treatment for osteoarthritis is physical therapy including physical examination, intervention, and rehabilitation to improve a patient's physical function. Modalities of physical therapy include manual therapy, balance and perturbation therapy, strength training, aquatic therapy, shockwave therapy, thermotherapy, laser therapy, therapeutic massage, and therapeutic ultrasound. In manual physical therapy, there is a focus on keeping the joint in motion rather than immobilizing it. It has been reported that this is an effective procedure for healing injured joints, as it can increase the flow of nutrients and growth factors to the articular cartilage and can stimulate the mechanical activation of tissue remodeling pathways (Saxon et al., 1999). In a study by Deyle et al. (2000), significant improvements were apparent in physical therapy groups, compared with placebo groups regarding walk times and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) scores. The WOMAC Osteoarthritis Index is a commonly used index for the evaluation of hip and knee stiffness, pain and physical function in OA. Another study published by Fitzgerald et al. (2011) investigated the effectiveness of physical therapy balance and perturbation therapy. This study produced positive improvements in WOMAC scores and self-reported pain and stability scores. Finally, in a study evaluating aquatic physical therapy (Hinman, Heywood, & Day, 2007), 71 volunteers with hip or knee OA underwent 6 weeks of aquatic physical therapy. There were significant improvements in pain and physical function in over 2/3 of the treated patients, compared to less than 1/5 of the control patients displaying significant improvement.

Pharmacological management approaches of OA often include NSAIDs, opioid

analgesics and viscosupplementation. Aziz et al. (2019) compared the use of NSAIDs with or without supplementation and intra-articular hyaluronic acid injection (viscosupplementation), for the alleviation of pain in OA knee patients. In an osteoarthritic joint, both synovial fluid integrity and viscosity are negatively affected. Because many patients experience side effects while taking NSAIDs, based on their history, some were given additional supplementation such as gastro-protectants or cardiovascular protectants (gastrointestinal protective agents are commonly included with long-term NSAID therapy). The group concluded that intra-articular hyaluronic acid injection proved more effective in pain relief than both NSAID treatment groups (Aziz et al., 2019).

Corticosteroids are one of the more popular pain management treatments for patients with OA. McAlindon et al. (2017) tested the use of corticosteroid injections within the articular cartilage as a treatment for OA. It was hypothesized that these corticosteroids could treat synovitis, which is known to be associated with OA, and slow disease progression. At the end of the study, it was determined that the intra-articular steroids did not prevent the disease but accelerated the degradation of cartilage; patients receiving steroid treatments had less measured cartilage than the control group (Mandl, 2019). In a double-blind study, patients with knee OA were given one injection of an extended-release formulation of triamcinolone (a corticosteroid) or placebo and asked to score their pain over 12 weeks. There was a significant improvement in pain scores in the treated group for the foremost weeks of the study and a gradual decrease in this improvement after 12 weeks, which led to the conclusion that this injection was effective

for quick, short-term pain relief (Conaghan et al., 2017). Another study compared three specific types of corticosteroid injections, methylprednisolone, betamethasone, and triamcinolone. The results of the study concluded that methylprednisolone and betamethasone were more effective treatments overall (Datta & Upadhyay, 2011). Intra-articular corticosteroids inhibit prostaglandin synthesis to reduce pain and control inflammation by down-regulating collagenase enzymes. They are relatively inexpensive compared to their surgical counterparts and are easy to administer in an outpatient setting, making them a popular choice for patients. These pharmacological treatments, although effective, do not repair damage to joints or prevent the damage from occurring in the first place. They are preferred as the last solution before surgical intervention in most cases (Arif et al., 2019). Additionally, older people with OA may benefit less from injections than younger individuals (Chen et al., 2019).

Surgical intervention is often thought of as a last resort for those suffering from OA. Methods of surgical intervention for OA include arthroscopy and total or partial joint replacement surgeries. Arthroscopy is an outpatient procedure during which an arthroscope, a small tool that has a camera and light, is inserted to less-invasively assess the joint issue. Debridement, suturing, flushing inflammation-inducing debris and crystals, resection of over-proliferative synovium, cutting away and removal of articular cartilage chips/fragments, and grinding down of osteophytes. can be accomplished during an arthroscopy (Felson, 2010). Sterile fluid is also pumped into the joint to widen it and make diagnosis and procedures easier. In a study involving 122 patients with knee OA who underwent arthroscopy concluded, using the Knee Society pain score, which is a

worldwide measure of pain in the knee, there was an average increase of 11.9 out of 50 points. This study did advocate arthroscopy as having a role in the therapy of OA. Two randomized clinical trials (RCTs) have shown no additional benefit to arthroscopic debridement over nonsurgical management in patients with moderate to severe knee OA (Kirkley et al., 2008; Moseley et al., 2002).

Total and partial replacement surgeries can be accomplished on most joints affected by OA. Complete joint reconstruction or replacement surgery is also known as arthroplasty. The total hip replacement surgery was the first successful joint replacement surgery and was pioneered by Sir John Charnley in 1967 at Wrightington Hospital in Lancashire, UK. During a total joint replacement surgery, parts of the damaged or arthritic joint are replaced with a prosthesis that is cemented to the healthy portion of the bone (Waugh, 1990).

A study by March et al. (1999) compared the quality of life in patients before and after joint replacement surgery. This study demonstrated improvement for patient's post-surgery, particularly in function and pain. Another study also compared pre- and post-operative outcomes using WOMAC scores, with a reduction in pain of 71% and 53%, a reduction in stiffness of 55% and 43% and an enhancement in physical function of 68% and 43% in the knee and hip OA patients, respectively (Bachmeier et al., 2000).

OA is a multi-factorial condition within the musculoskeletal system. Mesenchymal stromal cell (MSC) treatment has been considered by various scientists to be a possible solution for repair of damaged tissues because the MSCs can potentially differentiate into specific tissue types, including musculoskeletal tissues (Harrison-Brown

et al., 2019). Mesenchymal stromal cells can be obtained from bone marrow, umbilical cord blood, adipose tissue, peripheral blood, synovium, and periosteum. The cells are then cultured for isolation and expansion of the desired mesenchymal cell-types through environmental manipulation. They are a minimally invasive alternative to surgery for the remedy of degraded cartilage and damaged bone. Mesenchymal stromal cells have also been shown to have immunomodulatory properties making them a prime option for research into OA treatment. There have been many studies published using various models for MSC research in OA patients. These include horses, goats, sheep, rats, rabbits, pigs, guinea pigs, and mice. Mesenchymal stromal cells have an advantage over many other forms of treatment because, in addition to pain relief and increased joint mobility, they can potentially contribute to cartilage and meniscus tissue growth. As of now, there have been no serious side effects of MSCs published. The immunomodulatory effects of transplanted MSCs are well documented. However, very little is known about any direct contribution of transplanted MSCs to tissue regeneration. (Harrison-Brown et al., 2019).

### **Models**

Animal models are used in preclinical research to observe disease pathogenesis in real-time and to test various routes of disease prevention, deceleration, and treatment. Many factors contribute to the selection of an animal model for the disease. This can include relative size, reproductive rate, anatomical similarities to humans, genetics, the type of experiment/study, husbandry costs, social concerns (most commonly with



companion animals), and ease of handling. Within the discipline of OA research, many types of models have been used to further the understanding of the disease and treatments. These include the mouse, rat, guinea pig, rabbit, dog, sheep, goat, pig, and horse (Teeple et al., 2013).

### **Rodents and Other Small Mammals**

Many studies have used rodents and other small mammal models for OA research due to the shorter lifespan and therefore quicker aging and maturity development, availability of specialized strains, ease of handling due to small size, and low-cost (Poulet, 2016). These animals are often used as surgically induced models of OA, with increased rates of disease progression (Teeple et al., 2013). Data from these animal trials can provide a foundation for research to be done in larger animal models and even humans. However, a common concern when using rodent and other small mammal models is the significant differences in anatomy and physiology from humans and other models. Some of the more widely used small mammal models include ligament transection and meniscectomy in the mouse, partial menisci resection model in rabbits, ACLT and medial meniscal manipulation in rats and guinea pigs, and genetically modified mice (Ameye & Young, 2006; Bendele, 2001).

### **Canine Model**

The canine model is often used in orthopedic research. The canine ACLT model is very popular among joint-disorder researchers. Other models include the canine groove model, in which grooves are surgically made in the articular cartilage to induce disease,

the trans-articular impact model that induces subchondral bone trauma and partial or total meniscectomy (Ameye & Young, 2006; Bendele, 2001). The beagle breed is commonly used due to its thick cartilage and gives a better opportunity to histologically measure the depth of lesions (Bendele, 2001). One issue noted by Dr. Bendele in an ACLT, was the dog's tendency to carry an ACLT limb and it was assumed that this was due to a perceived instability factor inherent to the dog. This could be a factor in the decreased appearance of lesions in those favored joints due to the decrease in load-bearing of the treated joint.

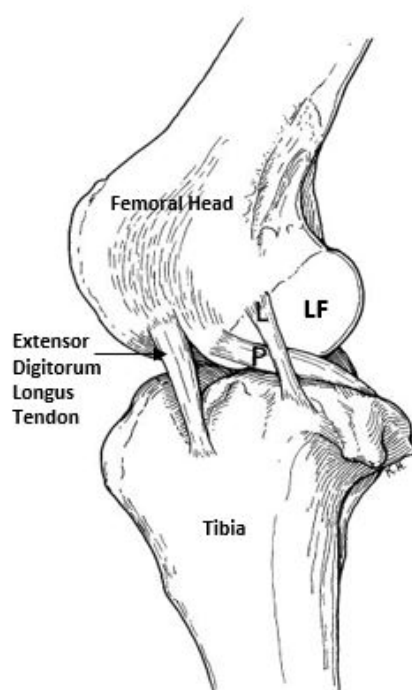
One of the most prevalent issues in using dogs in any form of research is the emotional and legal aspects. Over time, many researchers have come to agree that, although appearing often in a clinical setting, dogs are not necessarily the best models for orthopedic research and their advantages are analogous with more accessible models such as sheep and goats (Martini, Fini, Giavaresi, Giardino, 2001).

### **Sheep Model**

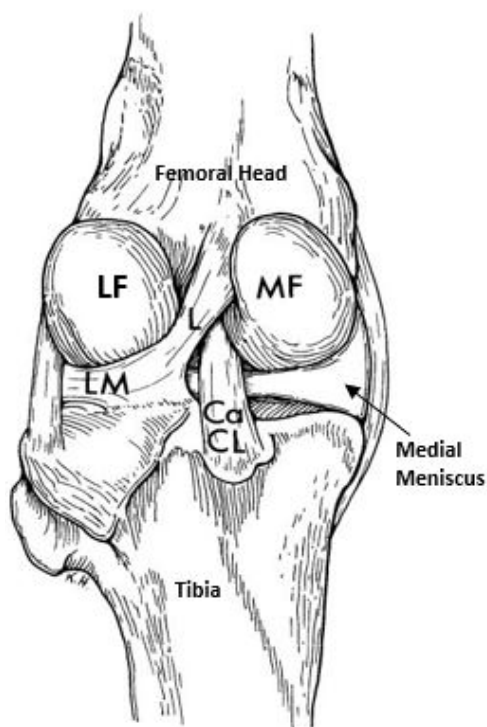
Sheep are a popular alternative to dogs in orthopedic research. They are docile and easy to handle due to their optimal size. They are used in orthopedic research for many kinds of disorders such as fractures, osteoporosis, limb lengthening, ligament repair, and OA. Sheep are large enough to allow for frequent sampling, unlike smaller models, and are robust enough to undergo multiple experimental procedures. Sheep reach sexual maturity within a year of birth, but their skeletal system continues to grow and develop after puberty. In many breeds of sheep, the growth of the iliac crest does not close until five years of age (Martini et al., 2001).

The ovine femoropatellar or stifle joint is, in many aspects very similar to the human knee. It is a complex joint including three bones and functions in a complete six degrees of freedom (Rosvold et al., 2016). This means the joint is free to move forward/backward, up/down, and left/right. The major bones involved are the femur, patella, and tibia. The stifle is a synovial joint and is often the largest in the body. It is the most commonly injured joint in sheep and a good model for osteoarthritis.

The sheep stifle has an intricate network of tendons, ligaments, and cartilaginous surfaces (Figures 5 and 6). There are two major condyles, the medial femoral and lateral femoral condyle. In general, the lateral femoral condyle is the larger of the two condyles. However, the medial condyle resides deeper than the lateral (Allen, Houlton, Adams, &



*Figure 5.* Schematic illustrations of the lateral view of the stifle joint. The (P) popliteal tendon passes deep to the (L) lateral collateral ligament and inserts on the craniolateral aspect of the lateral femoral condyle (Allen et al., 1998).



*Figure 6.* Schematic illustration of the caudal stifle joint. The (L) meniscomfemoral ligament originates on the (LM) lateral meniscus, passes caudal to the (CaCL) caudal cruciate ligament, and inserts on the (MF) medial femoral condyle (Allen et al., 1998).

Rushton, 1998). These condyles lie at the end of the femur and articulate with the cartilage-covered medial and lateral tibial plateau. The medial tibial plateau is the first and main site of damage in most stifle joints. It is often a main site observed to detect bony changes due to orthopedic disease (Holland et al., 2013). These articulating surfaces, along with the patellar periphery are the sites where signs of OA are most commonly found.

The major ligaments in the stifle include two cruciate ligaments (cranial and caudal), two menisci (medial and lateral), two meniscotibial ligaments (cranial and caudal), the transverse ligament of the stifle, and the meniscomfemoral ligament. The

cranial (CrCL) and caudal cruciate ligaments (CaCL) mirror each other originating from opposing condyles—the CrCL from the lateral femoral and the CaCL from the medial femoral. These ligaments play a crucial role in the stability and function of the stifle joint. The two menisci function as fibrocartilaginous pads around the articular cartilage that inhabit the space between the femoral condyles and the tibial plateau. The menisci have various secondary tendons and ligaments which provide additional support to the joint. The medial collateral ligament attaches to the medial meniscus and the popliteal tendon crosses in between the lateral meniscus and lateral collateral ligament (Allen et al., 1998). The meniscomfemoral ligament originates from the femur and passes between the two adjacent condyles to secure to the lateral meniscus. Anatomy and procedures commonly targeted for induction of OA include transection of the CrCL, transection of the medial collateral ligament, and partial or total meniscal meniscectomy/injury (Little et al., 2010).

### **Other Large Animal Models**

In addition to the ovine model, other large animal models such as the goat, pig, and horse have been used in orthopedic research. The pig, although similar in some aspects of physiology, has proven less ideal of a model due to quick body growth and excessive weight and issues in handling (Martini et al., 2001). If one chose to use the porcine model, one could purchase smaller models (minipigs, etc.), However, they are more expensive and still can present handling issues.

Horses are popular in orthopedic research, but not necessarily for application in human disease. The equine sport, work, and recreational industry can be, in and of itself, an end goal in orthopedic research. Injuries and disorders common in equine medicine,

such as OA, are popular areas of research due to the potential economic return on an effective treatment or cure. Although still used as a model for human research, the horse is more commonly used as a model for equine disease. Horses are an expensive large animal model. Their orthopedic anatomy is similar to humans, but they are difficult to manage due to their large size. They can be trained, but from the perspective of a researcher, this takes increased time and money as well. There are also social/cultural concerns when using horses as they are often (but not always) considered a companion animal species. There are benefits to many of the various models used in orthopedic research. Ease of handling, body size, similarities to human anatomy and robustness of sheep set them apart as an ideal candidate.

### **Trauma Effect**

When an articular joint is subjected to trauma, there are various compromising effects. These consequences may include chondrocyte death, accumulation of blood within the joint, and initiation of an inflammatory process (Lieberthal et al., 2015). Joint trauma can disrupt the specialized collagen matrix within the ECM and can lead to the fissuring of cartilage and the condensation of subchondral bone (Bhosale & Richardson, 2008). As the cartilage erodes, the bone beneath erodes as well (Lacourt et al., 2012). In addition, intense trauma can inhibit proteoglycan production from chondrocytes and often results in a very fibrous collagen type 1 repair of the injury.

### **Sports and Osteoarthritis**

Although sports participation has many health benefits such as preventing obesity,

depression, and other chronic diseases, there are risks as well (Sandmark & Vingård, 2007; Saxon et al., 1999; Tran et al., 2016). Many experts argue that elite sports are the only type of sports that pose significant orthopedic risk to the participant, while others say it depends on the amount of time spent and still others disagree that there isn't any significantly increased risk for athletes in the development of OA (Poulet, 2016). In a meta-analysis and review published by Tran et al., the increased risk for the development of OA did not come from simply participating in sports, but for those that were injured while participating in sports (Tran et al., 2016). This is particularly true among women (Spector et al., 1996). Further research revealed that retired professional male football players had a 2 to 3 times higher likelihood of having knee pain, radiographic knee OA and a total knee replacement, compared with males in the general population (Fernandes et al., 2017). In a similar study, active males with high exposure to sports had a 4.5 times higher likelihood of developing OA in the hip, compared with randomly selected, age-matched controls (Vingård, Alfredsson, Goldie, & Hogstedt, 1993).

Joint injuries, with and without surgical intervention (sport-related and nonsports related) increase the risk of developing OA (Saxon et al., 1999; Simon, Grooms, & Docherty, 2019). Sudden and intense impact or repetitive damaging impact can cause lesions in articular cartilage, leading to the degeneration and death of cells (Bhosale & Richardson, 2008). If the injury affects the subchondral bone, peri-articular soft tissues or the articulating function of the joint, it can decrease the capability of the joint to dissipate impact forces. These shifts in joint capability can lead to the initiation of OA symptoms, including cartilage damage, sclerosis of the subchondral bone and the formation of cysts

and osteophytes (Saxon et al., 1999). Some of the most frequently reported orthopedic injuries are anterior cruciate ligament (ACL) tears or ruptures. In the U.S., greater than 100,000 cases occur every year (Wang, Mitroo, Chen, Lu, & Doty, 2006). These injuries are also the most likely injuries to increase the risk for OA, compared with other common injuries (Tran et al., 2016). The ACL will not heal on its own due to the lack of vascularization. Those affected must undergo immediate surgical repair to ensure a more satisfactory recovery (Wang et al., 2006).

Poor management and rehabilitation of sports injuries also increase the risk of developing OA. Injury to a specific joint often creates a need for over-compensation by contralateral/other joints and increases stress on these joints increasing their risk for injury and subsequent OA (Saxon et al., 1999).

Preclinical research has used animal models of surgically induced injury or other methods of joint injury to study OA. The sheep has been a felicitous model for numerous studies. Induced ovine joint injury facilitates the progression of OA much more quickly than OA progresses in humans (Christiansen et al., 2012; Rosvold et al., 2016). Common methods used to create a trauma animal model for osteoarthritis research include disruption or transection of the meniscus and/or anterior cruciate ligament, intraarticular fracture, joint impact loading and the cartilage groove method (Roemhildt et al., 2010).

### **Ligament Desmotomy**

Ligament desmotomy is commonly used for the induction of OA. Targeted ligaments include the medial collateral ligament (MCL) or ACL/CrCL in rabbits, dogs, sheep, mice, and rats. Spontaneous injury of the MCL and ACL account for 95% of all



multi-ligament injuries in the knee joint in humans (Funakoshi et al., 2007). The ACLT or the Pond-Nuki Model was developed by Dr. M. J. Pond and Dr. G. Nuki at the University of Glasgow Veterinary School in 1973. A study published on rabbits by Z. Liu et al. (2016) used an MCLT and ACLT to compare their effectiveness as OA models. There was significantly more evidence of OA development in rabbits that underwent ACLT compared with those that underwent MCLT or controls (Z. Liu et al., 2016). When an ACL is injured, it often results in joint instability, which itself can lead to OA (Saxon et al., 1999). In human patients who incur an ACL injury, there is a 60% to 80% chance that they will develop OA within 20 years (Saxon et al., 1999). In ACLT rats, there was a significant amount of degradation in cartilage after 12 weeks (Silva et al., 2018). Additionally, when major ligaments are transected, there can be adverse effects on the remaining uncut ligaments (Funakoshi et al., 2007). Finally, in a pilot study at Utah State University published by Hill et al. (2017), sheep that underwent a Cranial Cruciate Ligament Desmotomy (CCLD) displayed decreased joint space, increased overall joint width, increased aggrecanase activity, and increased urinary GAG content, and other OA symptoms (changes in gait, soundness, etc.).

### **Joint Loading**

Another method for the induction of OA is impact joint loading. These methods are considered less invasive than direct surgical manipulation (Poulet, 2016). Commonly, joints in full flexion receive one or more swift strikes to specific joint regions to induce acute damage and exacerbate the onset of the disease.

The most commonly used joint for this type of induction is the stifle (knee or

femoropatellar joint; Poulet, 2016). The impact of obesity on the progression of OA further confirms that excessive loads on joints can produce an effective OA model (Roemhildt et al., 2010). Another advantage of the joint loading model is that the weight, distribution, and repetitions of loading are quantifiable making the comparison of severities less variable (Poulet, 2016). A study by Ewers et al. (2002) subjected Giant Flemish rabbits to impact loading of the patellofemoral joint. Experimental rabbits developed osteophytosis and increased bone thickness on the impacted joints, compared with the contralateral joints (Ewers, Weaver, Sevensma, & Haut, 2002). Mice subjected to tibial compression loading display significant increases in bone volume and Osteoarthritis Research Society International (OARSI) scoring 56 days post-injury (Christiansen et al., 2012). In cattle, impact loading resulted in significant joint degradation, compared with normal controls (Radin & Paul, 1971). In a study by Y. Liu et al. (2014), mice were given food in different size pellets to either increase or decrease the amount of mastication needed to consume them. Larger pellets meant more chewing while smaller pellets decreased chewing effort. Results indicated that the smaller pellet diet reduced cartilage thinning and degradation, decreased expression levels of collagen II and aggrecan, decreased the loss in subchondral bone and enhanced osteoclast activity.

### **Cartilage Groove**

An additional model for the induction OA is known as the groove model, where cartilage is damaged by drilling small grooves in the cartilage (without injuring the subchondral bone) to exacerbate the onset of orthopedic disease. Grooves are commonly

made in the weight-bearing portions of femoral condyles of the knee. This method is often used in animal models such as the dog, rat, and sheep. There is a decrease in cartilage thickness and an increase in trabecular bone thickness over time in rats who have undergone a groove procedure (Visser et al., 2016).

In a study by Marijnissen et al. (2002), a canine groove model was paired with immobilization of the contralateral limb to the trunk of the dog to ensure weight-bearing. This procedure resulted in an increase in inflammation within the synovium, a 77% increase in serum GAG content, and a greater Mankin score (a standardized criterium for grading cartilage) 20- and 40-weeks post-surgery, compared with controls. This method was repeated by Mastbergen et al. (2006) and again by Intema et al. (2007) with similar conclusions. A similar study in sheep, including the groove model and immobilization, resulted in damage very similar to the previously mentioned canine models (Mastbergen, Pollmeier, Fischer, & Lafeber., 2008).

### **Chemical Injury**

Another model for the induction of OA is the use of chemical injury. The use of monosodium iodoacetate (MIA) is well-established. Monosodium iodoacetate is an inhibitor of metabolic pathways and can cause cell death. It has been associated with a reduction in the number of chondrocytes and alterations of the articular cartilage and subchondral bone, mirroring OA in humans (Guzman, Evans, Bove, Morenko, & Kilgore, 2003). Rats who received a single injection MIA into the cartilage of the femoropatellar and tibiofemoral compartments of the stifle joint were shown to have a higher OARSI score over time through a histological analysis than controls (Takahashi,

Matsuzaki, Kuroki, & Hosono, 2018). Morais et al. (2016) also conducted research on rats using a similar chemical injury method. A placebo control group was injected with saline and a treatment group injected with MIA. Rats underwent forced exercise routines and a weight-bearing assay (Von Frey Test) in addition to injections and the results displayed a significant difference between groups with signs of OA greater in treatment groups than in placebo-treated controls (Morais et al., 2016).

### **Hormonal Influence**

Hormone signaling is critical for bone remodeling. Therefore, any increase or decrease in hormone signaling in mature bone will affect bone remodeling and likely be pathologic. Some of the main hormones involved include growth hormone, parathyroid hormone, estrogens, progestogens, androgens, leptin, serotonin, calcitonin, and thyroid-stimulating hormone. Growth hormone plays a major role in bone growth until adulthood. Parathyroid hormone (PTH) stimulates osteoclasts to reabsorb bone mineral, liberating calcium into the blood, enhances absorption of calcium from the small intestine, stimulates the production of the active form of vitamin D in the kidneys and decreases calcium loss in the urine (O'Brien et al., 2008). Estrogens contribute to skeletal maturation until the end of puberty and contribute to skeletal system maintenance post pubertally (Almeida et al., 2017). Leptin helps to maintain the homeostasis of osteoblastic and osteoclastic activity (Yadav et al., 2009). Serotonin decreases the actions of osteoblasts, thereby inhibiting bone formation. However, depending on where it is synthesized, serotonin can also be a stimulator of bone growth (Ducy & Karsenty, 2010).

Both calcitonin and thyroid-stimulating hormone oppose the action of PTH by decreasing osteoclast activity. Age-associated hormonal changes differ significantly between men and women.

Before the age of 50, OA is more common in men. However, as the population ages further, OA risk evolves to a point where most of those suffering from OA are women (Sniekers, Weinans, Bierma-Zeinstra, Leeuwen, & Osch, 2008). Trabecular bone is highly responsive to sex steroids and is regulated differently in males, compared with females and is also regulated differently between actively growing and mature females. Unlike in males, regulation of bone in females is subject to periods of reproductive cycling, pregnancy and an abrupt change in hormone signaling at menopause. During pregnancy, trabecular bone is rapidly mobilized for mineralization of the fetal skeleton (under the influence of high levels of estrogen and progesterone) and because of its increased surface area, trabecular bone in females is more sensitive to change.

It is hypothesized that this shift in OA risk is due to the changes women go through during the menopausal transition (Roman-Blas, Castañeda, Largo, & Herrero-Beaumont, 2009; Sniekers et al., 2008; Turner, Athanasiou, Zhu, Alvis, & Bryant, 1997). The menopausal transition affects many aspects of a woman's overall health and functions. When young ovarian function is restored in aged female mice through the transplantation of young ovaries, there is a significant decrease in osteophytosis (Mason, Terry, Merchant, Mason, & Nazokkarmaher, 2015). Hormonal changes begin in pre-menopause, continue through perimenopause and normalize in the post-menopausal stage. During the menopausal transition, cyclic estrogen and progesterone delivery

become unreliable and this leads to increased bone remodeling. Rapid increases in bone resorption outpace increases in bone formation, producing a deficit in bone replacement (Matsui et al., 2015). Estrogen deficiency has been linked to osteoporosis and low androgen and estrogen have been linked to high-turnover osteopenia (Almeida et al., 2017).

## **Estrogen**

Because much of female OA occurs during and after ovarian changes, it is suggested that there is an OA-protective effect of estrogen. The three main estrogen types are estrone, estradiol, and estriol. Both  $\alpha$  and  $\beta$  estrogen receptors have been identified in many tissues within joints, including cartilage, bone, fat, ligaments and the synovium (Sniekers et al., 2008; Yang, Kim, Lim, & Oh, 2012). In a review by Sniekers et al., 11 studies supported the observation that ovariectomy (OVX), and, therefore, the silencing of a major cyclic estrogen source proved detrimental to cartilage tissue homeostasis, ranging from fibrillation to degradation to complete cartilage loss. An ovine OVX model displayed a lower measure of compressive stiffness in cartilage than in control sheep (Turner et al., 1997). In a study by Miyatake et al. (2016), OVX mice who completed a forced exercise routine displayed a greater amount of OA compared with sham surgery mice, or sham surgery mice that completed a forced exercise routine.

Various animal models and procedures are used to detect the effects of estrogens on OA development and progression, with one of the most common being the OVX (Sniekers et al., 2008). Another method often used is estrogen replacement therapy (ERT). There are some contradicting results with the use of ERT, but this could be due to

a difference in procedures and methods between studies (Ham, Loeser, Lindgren, & Carlson, 2002). In the study by Ham et al., OA lesions were less extreme in adult female macaques who received a regimen of ERT, compared with a control group. In OVX rats, animals that received a combination of estrogen and progesterone therapy fared better than estrogen alone, progesterone alone or no hormonal therapy groups when evaluated using the OARSI system. (Yang et al., 2012). However, other research/reviews contradict the presence of a positive estrogen effect for OA (Hanna, Wluka, Bell, Davis, & Cicuttini, 2004; Mahajan & Patni, 2018; Mahajan, Tandon, Verma, Sharma, 2005). It has even been suggested that estrogen is detrimental to cartilage homeostasis (Turner et al., 1997).

Menopausal and young ovariectomized women and animals have a higher prevalence of OA than women with fully functional (cycling) ovaries. Still, there are more changes than just a decrease in estrogen that a woman's body goes through when she undergoes menopause, or when the ovaries are removed. Although estrogen is probably a player in the onset and development of OA, it has not yet been determined if its lack or replacement exacerbates or improves the disease.

### **Progesterone**

Progesterone is another endogenous sex hormone mainly involved in pregnancy and fertility in women. It is just one of the types of progestogens within the body. Its role in bone strength is considered minor compared to its reproductive functions. In the study mentioned previously by Yang et al. (2012), the solo estrogen replacement therapy was inferior to the combined estrogen and progesterone replacement therapy in suppressing

the progression of OA. This was particularly true when it came to bone and cartilage turnover. Additionally, combined therapy of estrogen and progesterone has been used to treat osteoporosis after menopause (Yang et al., 2012). Research on progesterone's influences on OA has been limited. It is still unclear whether it plays a major role in the pathogenesis of bone and joint disease.

### **Androgen**

Androgens are another candidate for influence in OA. The two main androgens are testosterone and androstenedione. Other common androgens include dehydroepiandrotestosterone (DHEA), DHEA sulfate (DHEA-S), and dihydrotestosterone (DHT). Androgens have an influence on the skeleton's growth and maintenance. Both estrogen and androgen deficiency cause a loss of cancellous and cortical bone mass (Almeida et al., 2017).

Sex hormones contribute to the development of osteoporosis (OP; Almeida et al., 2017). Sex hormones also play an important role in the development of OA in mice. Castration decreased OA in male mice, compared with control males, contrasting the results in females who had undergone an OVX. Adding exogenous DHT created more severe OA in males mirroring the male control mice. Interestingly, overall, male mice developed more severe OA than female mice, however, the female mice were generally less active, which may have been a factor (Ma et al., 2007). In a case-control study involving 573 women between the ages of 24 and 45, a decrease in testosterone was significantly associated with an increase in Kellgren-Lawrence scores (Kellgren Lawrence grading system is a commonly-used radiological classification of



osteoarthritis) within the hand (Sowers et al., 1996). Compared to other pathways for OA, androgens have not been studied as fervently. Indications from current research show that androgens could play a role in the development and severity of OA.

### **Forced Exercise Effect**

Exercise can increase stress on joints and bones, but without additional additive measures, induction of orthopedic disease often takes an extended period to develop. Pairing forced exercise with surgical or genetic manipulation has the potential to create more severe disease and at a quicker pace (Poulet, 2016). Athletic people are more prone to both normal wear and tear and injuries to their skeletal system. In bone-related disease modeling, forced exercise may be paired with other treatments or used solo to exacerbate disease progression (Appleton et al., 2007; Miyatake et al., 2016).

Rodents are a popular choice in exercise models (Poulet, 2016). Rats and mice that were run throughout their lives were more likely to develop severe OA, compared with controls (Lapveteläinen et al., 2002; Lapveteläinen et al., 1995;). Rats that underwent forced exercise also suffered from an increased speed of OA progression (Appleton et al., 2007). Lubricin, a glycoprotein secreted in the synovial joint for lubrication and smooth function, was significantly decreased in ACLT rats who were forced to exercise, compared to unexercised ACLT rats (Elsaid et al., 2012; Jay & Waller, 2014). In a study on the manifestation of OA in sheep that underwent CCLD combined with oblique-angle forced exercise, there was a significant increase in bone joint width and osteophytosis within joints, compared to unexercised control sheep.

Aggrecanase activity, a good indicator of cartilage degeneration, increased at a greater rate in exercised CCLD sheep, compared with unexercised CCLD sheep (Hill et al., 2017). Forced exercise is an effective method for the exacerbation of OA with significant supporting evidence for its use in the study of the pathogenesis and treatment of OA.

### **Degradation of Connective Tissues**

The natural degradation of connective tissues with age is facilitated by the very active remodeling environment in which they reside. Tissue homeostasis can be disrupted by inactivity or overactivity of several factors within and outside of the matrix.

#### **Articular Cartilage**

The active remodeling of articular cartilage is a normal and necessary process. The ability of cartilage to endure heavy impact and maintain resistance is dependent upon its structural soundness. Cartilage degradation is often manifest by the destruction of its ECM, which is the foundation of its resistive forces. Destruction of the ECM is often spurred by overactive proteolytic enzymes, commonly by the upregulation of MMPs. One of the most highly expressed MMPs in cartilage is MMP-3, which can degrade many proteoglycans (Peffer, Thornton, & Clegg, 2016).

During this degradative state, there is excessive loss of cartilaginous proteins, including collagens and proteoglycans, important components of the ECM. One critical molecule broken down during disease states is aggrecan, which is a major structural macromolecule of cartilage. Aggrecan contributes to water retention and provides resistance to compression and can make up as much as 10% (by weight) of the cartilage

matrix. Among the other molecules destroyed are cartilage oligomeric matrix protein and fibromodulin. Cleavage fragments of these damaged proteins cause further disruption by disturbing the function of chondrocytes within cartilage (Peffer et al., 2016). This disturbance further delays the synthesis and restoration of important ECM components.

### **Ligaments**

Ligaments are dense bands of connective tissue that form attachments between bone, tendon, and muscle. The ACL is a poorly vascularized tissue and therefore its capacity to heal is very limited without surgical intervention (Wang et al., 2006). The ACL, like cartilage, is characterized by an extensive ECM, which is chiefly type I collagen. Other components include other collagens, proteoglycans, decorin, tenascins, elastin, and fibromodulin. Within the ligament, collagen contributes tensile strength and proteoglycans provide resistance to compression and stress. When the ACL is degraded, there is an altered cell organization, death, proliferation, and differentiation (Nakahara et al., 2013). Another main characteristic of a degraded ligament is the presence of chondroid metaplasia, or cartilage-like abnormal changes in the tissue (Asahara, Inui, & Lotz, 2017).

### **Tendons**

A tendon is a band of connective tissue that attaches muscles to bone. Similar to ligaments, tendons have a limited ability to repair and respond to changes, such as overloading largely due to the lack of vascularization in this connective tissue. Like other connective tissues, tendons are composed of an ECM surrounding specialized cells.

These cells are called tenocytes and they are responsible for the synthesis and degradation of all the components of the ECM in which they reside. Overloading of tendons can lead to degradation. However, the mechanisms behind this process are still being studied. Changes in matrix-remodeling enzymes and altered gene expression may play a role. Specifically, TIMPs are key players in ECM remodeling and studies have shown that they could influence the degradation of tendons (Parkinson et al., 2010).

## **Muscles**

Muscle wasting (sarcopenia) is when muscles atrophy due to a change in physical and/or metabolic activity. It is common in geriatric individuals or those who have been injured or immobilized for a long period. Other causes include poor nutrition, age, and genetic factors. In the elderly, sarcopenia can contribute to inactivity, disability and even mortality (Roth, Metter, Ling, & Ferrucci, 2006). This loss in muscle not only decreases the volume, but also the quality of the muscle tissue (Ryall, Schertzer, & Lynch, 2008). Muscle strength and quality peak in a person's 30s and 40s, followed by a gradual decline throughout the rest of life (Roth et al., 2006). It is common for muscle wasting to occur in lower limbs affected by OA (Silva et al., 2018).

The cellular mechanisms behind muscle wasting may be associated with oxidative stress. There is an accumulation of intracellular damage over the lifespan that negatively affects muscle retention (Ryall et al., 2008). This accumulation leads to dysfunction of the mitochondria within the cells and skeletal muscle is notably prone to these effects (Hepple, Baker, Mcconkey, Murynka, & Norris, 2006). Other age-related factors can also contribute to the increase in muscle wasting including hormonal factors,

general health status, circulating growth factors, neuromuscular factors, inflammatory factors, and others (Habermehl & Mason, 2019; Ryall et al., 2008). One recognized negative mediator of muscle mass growth is myostatin. It has been hypothesized to promote protein degradation through the stimulation of a proteasome system that contains specific enzymes for skeletal muscle degradation. Myostatin has also been shown to debilitate the activation of satellite cells, which are critical for muscle growth and repair (Silva et al., 2018). Mutations in the GDF8 gene (myostatin) cause excessive muscle formation and is reported in some cattle breed such as the double-muscled Belgian Blue cattle (Kambadur, Sharma, Smith, & Bass, 1997).

### **Measurement Tools for Osteoarthritis**

#### **Gait Analysis**

Analysis of gait within human and animal studies can be a valuable tool in evaluating the function of joints, as well as the pain associated with injury and disease. There are many aspects of gait that can be measured including stance time, dynamic joint loading, maximum joint angle, ground reaction force (GRF), speed of gait, and stride length. A study by Deluzio and Astephen (2007) found steady declines in speed and stride length, significant changes in joint angles and flexion, and stance time in the hips and ankles of subjects with severe to moderate OA, compared with subjects with no OA. Subjects with OA also displayed a significantly slower gait than those without OA. Subjects with OA walking on the level ground had decreased knee flexion and male subjects with OA had an even lower knee flexion than females with OA (Kaufman, Hughes, Morrey, Morrey, & An, 2001).

Studies of gait analysis may use force plates, video recording of gait cycles, painted markers, motion tracking devices attached to the body and motion tracking devices surgically embedded in the animal. Force and width of gait measures (often used as indicators of pain) in rats that underwent an ACLT + MCLT were significantly decreased compared with controls (Sudirman et al., 2018). In dogs and cats with clinical signs of OA, there is a decrease in GRF, which is more pronounced in cases with knee OA rather than hip OA (Moreau, Lussier, Ballaz, Troncy, 2014). Hill et al. (2017) used video recording and painted markers to analyze the gait of sheep that underwent a CCLD and an oblique-angle forced exercise routine and sheep that only underwent an oblique-angle forced exercise routine. Measurements were taken on a large animal treadmill with controlled gait speed. A decrease in maximum stifle angle and range of motion was detected in the CCLD + exercise sheep, compared to the exercise-only sheep. Additional parameters from this study also supported the presence of clinical signs of OA (Hill et al., 2017). Gait analysis can be a good indicator of pain and stiffness due to OA or other musculoskeletal diseases.

### **Osteophytosis and Other Skeletal Changes**

The abnormal gain and loss, as well as the location of bone changes, can be key indicators in joint health. Clinical signs of OA include osteophytosis, osteopenia, sclerosis, joint space measurement and joint bone width (Teirlinck et al., 2019). These abnormalities are usually detected via radiograph, but other systems have been used including magnetic resonance imaging (MRI), bone density scans, computed tomography (CT) and microcomputed tomography (micro-CT).

Osteophytosis, also known as bone spurs, is excessive or abnormal bone growth, often at the periphery of normal joint structures, that is indicative of OA. It is a very common measurement and is often the first definitive sign of OA in radiographic analysis (Neogi, 2012). Osteophytosis can limit joint mobility and cause pain. Osteophytosis was observed in mice two weeks after OA induction using an injection of collagenase (Blom et al., 2004). Osteophytosis score correlated strongly with time after CCLD in dogs and rats (D'Anjou et al., 2008; Mason et al., 2017). Osteophytosis is a major component of the Kellgren-Lawrence grading scale of osteoarthritis in humans (Bastick et al., 2017). Osteophytosis was increased in sheep that were destabilized using a CCLD (Hill et al., 2017).

Osteopenia is a decrease in bone mineral density (BMD) where the mineralization of bone present is less than normal. Osteopenia is a hallmark of osteoporosis, which is strongly associated with OA and often occurs in conjunction with or precedes osteophytosis. There is often thinning of the bone adjacent to an osteophyte. Osteopenia is linked to the neovascularization characteristics of new bone formation. It has also been hypothesized that inflammation within the bone is related to the appearance of osteopenia (Buckland-Wright, 2004). Some research has actually suggested that OA and osteoporosis are inversely related (Diarra et al., 2007). Studies of the properties of bone from both types of disease displayed thickening of bone as a characterization of OA and thinning of bone as a characterization of osteoporosis (B. Li & Aspden, 1997). However, both are characterized by abnormal bone remodeling.

In contrast to osteopenia, sclerosis describes a hardening of the subchondral bone.

It usually occurs in the later stages of the disease and can be a marker for OA progression. When there is a loss in soft tissue such as cartilage, the bone beneath attempts to re-grow and increase its ability to resist trauma. On radiographic analysis, sclerosis appears significantly more radiopaque (brighter) than normal bone (Figure 7). Similar to osteophytosis, sclerosis correlated strongly with time after CCLD in dogs (D'Anjou et al., 2008). In a study by Bastick et al. (2017), patients had an increased

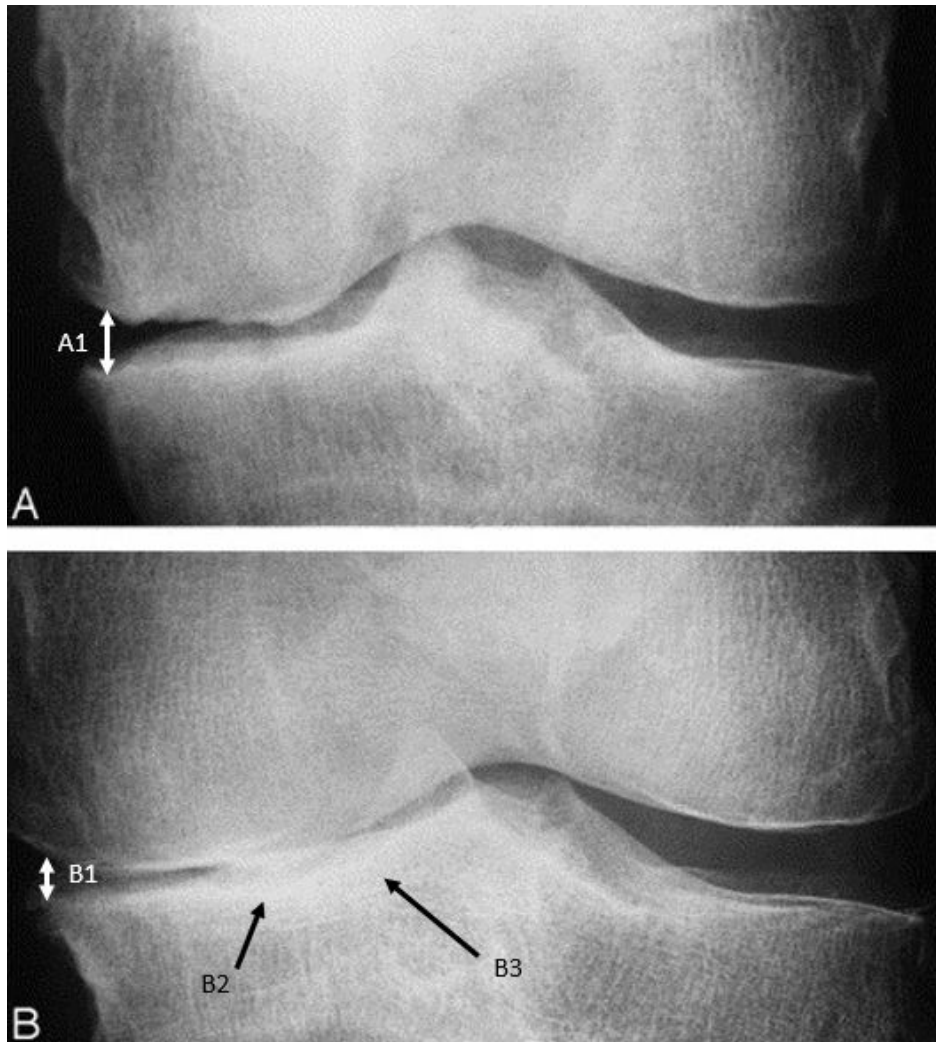


Figure 7. Radiographic images of knees with early OA (A) and definite OA (B). Sclerosis is present on the femoral and tibial plateaus of the bones in both images and there is severe joint space narrowing, in addition, more in the definite OA (Buckland-Wright, 2004).

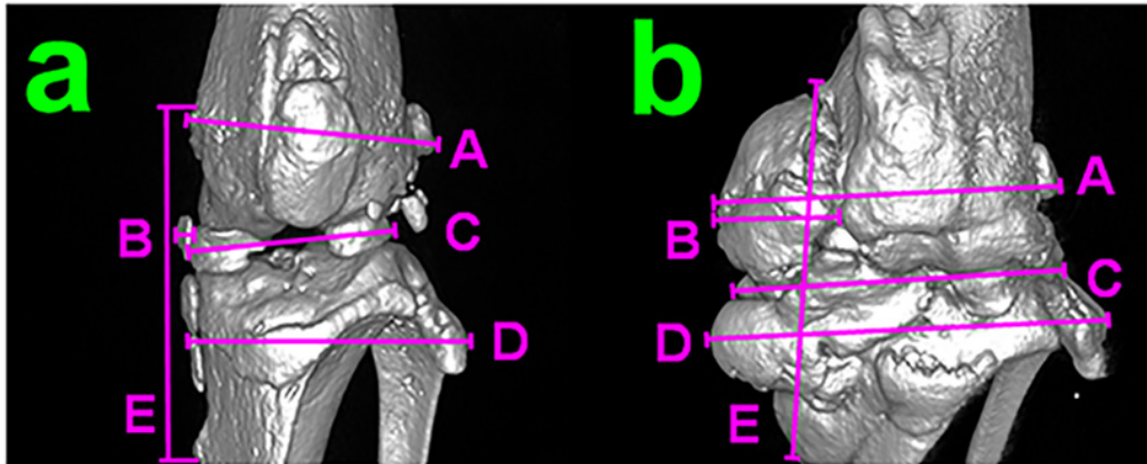


probability of needing total hip replacement surgery if they had been previously diagnosed with femoral subchondral sclerosis. In a similar study on elderly women, the presence of sclerosis also increased the likelihood of total hip replacement (Lane, Nevitt, Hochberg, Hung, & Palermo, 2004).

Joint space width is also a common radiographic measurement used in human studies as an indicator of OA progression. However, it can be unreliable in animal trials, as it is difficult to ensure an animal is fully weight-bearing and motionless unless anesthetized, which changes the normal positioning of the joint (D'Anjou et al., 2008). Joint space width is often used as an indicator of cartilage thickness. A decrease in cartilage thickness often indicates the degradation of the cartilage, which is highly correlated with the progression of OA. In patients with established OA, there is commonly a decrease in joint space, compared to those with early OA (Buckland-Wright, 2004; Figure 7). The study by Hill et al. (2017), established a decrease in joint space in sheep who underwent a CCLD combined with an oblique angle forced exercise routine, compared with controls.

A final common measurement tool for OA is joint bone width, the total width of mineralized tissue at the stifle joint. This measurement supports the diagnosis of osteophytosis as it is an additional measurement of excess bone growth (osteophytes and enthesophytes, abnormal bony projections at the attachment of a tendon or ligament, are often included in and contribute to this measurement [Figure 8]). Metacarpal joint width was increased in women with OA, compared to women without OA (Yahata et al., 2002). There was also a dramatic increase in bone width within the joints of destabilized sheep,

exercised sheep and destabilized + exercised sheep, compared with controls (Hill et al., 2017).



*Figure 8.* A depiction of joint measurements taken of a micro-CT scan of a rat stifle (a) Cranial view of a 3D reconstruction of micro-CT data from a joint with minimal perturbations. (b) Cranial view of a 3D reconstruction of micro-CT data from a joint with maximal perturbations. Measured parameters include: A- Distal femur width, B- Medial collateral ligament thickness, C- Joint width at the articulating surface, D- Proximal tibia/fibula width, E- Medial collateral ligament length (Mason et al., 2015).

### **Biomarkers of Disease**

Biomarkers of osteoarthritis can be used to track disease progression and severity. Because OA is a progressive disease, clinical signs do not usually manifest until well into the disease evolution. The use of biomarkers could be useful in identifying the disease in its early stages.

Aggrecan is one of the first proteins that is fragmented and released during the degradation of cartilage (Peffer et al., 2016). Generally, collagen loss does not begin until much of the aggrecan has already been degraded (Ravindra et al., 2018). The fragments of aggrecan can be used as biochemical markers for the indication of OA.

Matrix metalloproteinase-3 and ADAMTS-4/5 can cleave aggrecan, along with other proteoglycans at different sites, leaving behind characteristic fragments, indicative of the degrading enzyme. Because OA is a progressive disease, it is challenging to detect the onset or early development (Peffer et al., 2016). The use of protein biomarkers has proven useful in detecting early OA. One method for testing aggrecan content in serum is the chondroitin sulfate epitope 846 (CS846) enzyme-linked immunosorbent assay (ELISA). There is an increase in the aggrecan fragment/epitope CS846 within the synovial fluid of osteoarthritic horses. Horses with dorsal metacarpal disease displayed increased CS846 levels. There was also a gradual increase in total GAG levels in the study (Frisbie et al., 2010).

Glycosaminoglycans are important components within cartilage, especially for compression stiffness, tissue hydration and viscoelastic properties (Welsing et al., 2008). The concentration of GAGs in circulation has been shown to increase at the beginning of cartilage degradation (Frisbie et al., 2010). In guinea pigs, GAG increased within the synovial fluid of subjects that underwent an ACLT; these results correlated with an increase in the subject's Mankin scores (Wei et al., 2010). An additional study by Frisbie, Al-Sobayil, Billingham, Kawcak, and McIlwraith (2008) revealed an increase in serum GAG and CS846 in horses that were diagnosed with OA and subjected to exercise. Another method to detect circulating GAG is the use of the dimethylmethylene blue (DMMB) assay. This assay utilizes the conjugation of 1, 9-DMMB with GAGs to detect the concentration of GAG in serum and urine (Palmer, Bertone, McClain, 1995).

The use of aggrecan and GAG as osteoarthritis biomarkers has been reliable in

many studies. These biomarkers can be used as an additional indicator of disease progression within the joint. They can also be used to study the pathobiological processes in osteoarthritic joints (Frisbie et al., 2008).

### **Signaling Pathways**

Several molecular mechanisms play a role in bone formation, degradation, and repair. Studies have indicated an association of the Wingless-related integration site (Wnt) signaling pathway, Dickkopf-1 (Dkk-1) and sclerostin with OA progression.

#### **Wnt Pathway**

The Wnt signaling pathway, also known as the  $\beta$ -catenin signaling pathway, is critical for the development of many organs. It is also a major regulator in the repair and maintenance of many tissues. This includes the growth and remodeling of orthopedic tissues. The perturbation of this pathway has been associated with many diseases. Maintenance of homeostasis is achieved when there is a balance between active and inactive Wnt signaling. When Wnt is inactive,  $\beta$ -catenin is phosphorylated and degraded, and transcription of Wnt-responsive genes decreases. When Wnt signaling is active, its proteins cause the accumulation of  $\beta$ -catenin in the cytoplasm.  $\beta$ -catenin is then able to enter the nucleus to initiate the expression of Wnt target genes. These genes oversee stem cell renewal, differentiation, and intercellular communication. Wnt modulating molecules have been used to treat disease. Wnt signaling provides a pathway for the skeleton to adapt bone structure according to changing biomechanical needs (Amrein et al., 2014).

The Wnt pathway is crucial to balancing the process of progenitor cells

differentiating between osteoblasts and chondrocytes. Chondrocytes form cartilage and osteoblasts form bone. Increased Wnt activity in the subchondral bone and synovium drives the production of osteoblasts and proteases that degrade cartilage. This causes thinning of cartilage and an increase in bone growth in the form of osteophytosis.

Inhibition of the Wnt pathway may cause progenitor cells to become cartilage-forming chondrocytes, prevent osteophyte formation and block protease-mediated cartilage degradation (Diarra et al., 2007). Wnt pathway signaling is also significantly influenced by ovarian hormones (Manolagas, Obrien, & Almeida, 2013).

### **Dickkopf-1**

Additionally, antagonists of the Wnt signaling pathway such as Dkk-1, Frizzled-related protein (FRP) and Gremlin-1 within cartilage play an important role in OA pathogenesis (Leijten et al., 2012). Dickkopf-1 (Dkk-1) is a known inhibitor of Wnt signaling and influences skeletal development (Diarra et al., 2007; Weng, Wang, Ko, Sun, & Wang, 2010). It is a regulator in both humans and animals. Because active Wnt signaling is a major driver in bone growth, an inhibitor of this pathway is necessary to keep bone growth in check and maintain the process of bone remodeling. Although an inhibitor of Wnt signaling, Dkk-1 does not completely block Wnt signaling (Mason et al., 2017). There is an increase in BMD and bone formation in Dkk-1-knockout mice (Weng et al., 2010). This has been additionally described in mice that underwent attenuation of Dkk-1. There was a decrease in bone surface and trabecular bone volume (MacDonald et al., 2007). There is also an impairment in osteoblast matrix mineralization and osteopenia in mice that have an overexpression of Dkk-1 (Funck-Brentano et al., 2014). An increase

in endogenous circulating serum Dkk-1 slows the progression of hip OA in elderly women (Lane, Nevitt, Lui, Leon, & Corr, 2007) and the expression of Dkk-1 in chondrocytes inhibits cartilage destruction in OA (Oh, Chun, & Chun, 2012).

### **Sclerostin**

An osteocyte is a specialized bone cell that responds to mechanical stress within the skeleton (Lewiecki, 2014). Osteocytes can produce a specific protein known as sclerostin, which is important in the regulation of osteoblastic activity (Devarajan-Ketha, Craig, Madden, Bergen, & Kumar, 2012). Sclerostin can be used to inhibit the Wnt pathway and therefore bone formation and osteoblast differentiation (Matsui et al., 2015). This inhibition of the Wnt pathway makes sclerostin a key regulator in bone turnover (Stott et al., 2019). The formation of bone is influenced by many growth factors, such as bone morphogenetic proteins (BMPs) and Wnts, both of which are significantly influenced by sclerostin (Devarajan-Ketha et al., 2012). Sclerostin is also involved in vascular calcification (Amrein et al., 2014) and sclerostin expression is decreased after mechanical loading (Robling et al., 2007). There are two genetic disorders associated with a natural lack of sclerostin, sclerosteosis and Van Buchem's disease (Van Hul et al., 1998). These disorders lead to significantly increased BMD (Amrein et al., 2014). In addition to these diseases, a lack of sclerostin can also result in sclerosing bone dysplasia (Devarajan-Ketha et al., 2012). Sclerostin levels also increase with the progression of menopause (Matsui et al., 2015) and this could be evidence of an association between the sclerostin protein and a decline in bone formation with an increase in bone resorption.

## Gene Therapy

Gene therapy is a clinical/experimental technique that alters gene function *in vivo* to treat or prevent disease. The basic principle behind the therapy is to restore the body's faulty or deficient gene/gene expression in a one-time fix (Cross, 2019). The basic goal is to deliver the desired replacement of a specific gene/promoter/enhancer/inhibitor to the problem area or organ so that it can produce what is missing or defective for that disease or disorder. It is desired that the replacement be restricted to the target tissue. This approach has great appeal for its potential efficiency and accuracy (Peng & Russell, 1999). There are many genes at work within the function and maintenance of joints and many candidates for use in gene therapy solutions.

Bones and cartilage are two of the major tissues involved in OA. There are multiple factors in the correct and stable function of bone creation, remodeling, structure, and maintenance. These include MMPs, ADAMs, and ADAMTs among others. Within the MMP group, sub-families include gelatinases, matrilysins, collagenases, stromelysins and membrane-type MMPs (Javaheri et al., 2016). Interference or inhibition of MMPs has the potential to have a significant effect on both bone and cartilage function.

The TIMP proteins inhibit MMPs within the ECM. There are four TIMPs (1-4) and they are unique based on their levels of inhibitory abilities and molecular features. They have specificities in their inhibition of ADAMs and ADAMTSs (Jackson, Defamie, Waterhouse, & Khokha, 2017). The TIMPs contain two domains, the N-terminal and C-terminal with three disulfide bridges stabilizing each terminal (Woessner, 2001). The TIMPs have almost ubiquitous roles throughout the body, including embryonic somites,

lung, kidney cortex, liver, spleen, muscle, heart, brain, ovarian follicles, testis and hair follicles (Javaheri et al., 2016). Normally, TIMP activity will balance MMP activity to maintain homeostasis within tissues (Woessner, 2001). When this becomes out-of-balance, tissue destruction can occur.

Of the TIMPs, TIMP-3 has a specific suppressive ability against ADAMTS4 and 5 as well as downregulation of TNF- $\alpha$  converting enzyme (TACE). Tissue inhibitor of metalloproteinase-3 plays a key role in tissue remodeling, including bone (Javaheri et al., 2016). ADAM-7, a main regulator in bone development is inhibited by TIMP-3 (Gooz, 2010). In a study published by Mason Donahue, Yoskowitz, & Richardson (2013), cartilage biopsies were co-cultured with *TIMP-3*-stably-transduced HEK cells and recombinant adeno-associated viral vector (rAAV) *TIMP-3*-transduced equine fetal fibroblasts. The GAG content and aggrecanase activity were analyzed. There was an increase in GAG retained in the cartilage and a decrease in aggrecanase activity in samples that included TIMP-3 protein or the *TIMP-3* transgene (Mason et al., 2013). Tissue inhibitor of metalloproteinase-3 is the only one of the TIMPs that binds very strongly to the ECM. This is due to the interaction of heparan sulfate and chondroitin sulfate on cell surfaces or to secreted proteoglycans on the N-terminal domain. Because of this secure binding to the ECM, TIMP-3 generally remains local (although it can be detected in the circulation) and has a greater ability to inhibit specific ADAMTSs, MMPs, and ADAMs, and ADAMTS4/5, which are primarily responsible for aggrecan degradation in cartilage (Woessner, 2001). The *TIMP-3* gene could be a good candidate for gene therapy for the treatment, prevention or deceleration of OA.



## **Viral Vectors**

Viral vectors are often used in gene therapy due to their effective transfer of genetic information and their ability to be delivered to difficult areas of the body without disruption of the regular tissue configuration. There are a few strategies for modifying the binding properties of these viruses. One such strategy is genetically modifying the vector coat proteins of retroviral, adenoviral, herpesvirus, and other vectors. Another is using a soluble bifunctional crosslinker that binds to both the surface of the virus and the surface of the cell to create a molecular bridge. A combination of these approaches can be used, or the coat proteins can be hybridized with another serotype that already has the desired host range (Peng & Russell, 1999).

In gene therapy, there are three main types of viral vectors, lentivirus (LV), adenovirus (AdV) and adeno-associated virus (AAV). The LV can carry up to 30 kb of DNA. However, because LV inserts into the genome, it can cause high rates of insertional mutagenesis and has led to leukemia in children who were injected during a research study (Bokhoven et al., 2008). Adenovirus can carry the largest amount of DNA, up to 200 kb, but also inserts into the genome and produces an intense immune response (Cross, 2019). The highly immunogenic virus is used mostly in vitro (Ali, 1996). It has been known to cause respiratory, intestinal and eye infections in humans (Wold & Toth, 2013).

In 1999, a gene therapy trial was conducted by Dr. James Wilson at the University of Pennsylvania which produced an unexpected high immune response and the death of a patient in response to an adenovirus vector. This paused the industry of gene therapy for

years, but significant technical strides have been made recently and AAV has replaced AdV as the vector of choice for in vivo work (Cross, 2019). The adeno-associated viruses are infectious human viruses with no known disease association. However, these vectors can only carry a maximum of 4.7 kb of DNA, making them very specific and less efficient in delivery. Serotypes are created specifically for safe transfection with highly specific cell and tissue tropisms and a reduced risk of insertional mutagenesis. These vectors are mostly non-integrating and seldom insert their viral DNA into the chromosomal DNA of the host. These vectors are considered less immunogenic and can often be worked with at a Biosafety level 1 (Wold & Toth, 2013).

Most experimental and clinical AAVs are modified so that the genes for viral replication are removed. A therapeutic or marker gene along with suitable promoter/enhancer regions are inserted into the viral DNA and the virus will then cause the production of that chosen protein from transfected cells. Recombinant AAVs can infect both dividing and non-dividing cells, have an extensive host range, cannot proliferate, rarely causes a significant cell-mediated response within the host's immune system and generally can provide enduring episomal expression (Tomar et al., 2003). Because the virus cannot proliferate, it will dilute over time in the targeted tissue with the dividing of cells (Mason et al., 2015). Serotypes are created that contain specific tissue or cellular tropisms. The virus is contained within a capsid, which is a coating of proteins. This is what the target cell or tissue comes in contact with during infection. Common serotypes for rAAV vectors include serotype AAV1, AAV2, and AAV5 - AAV9. AAV1 has a capsid specific to the retina and pancreas and is very good at crossing blood barriers

(Ali, 1996; Mason et al., 2015). A study by Ali displayed the capabilities of AAV in transducing photoreceptor cells within the retina. Recombinant AAV2 is more commonly used for the liver and kidney tissues, however, humans have been shown to have strong neutralizing antibodies for this serotype.

Research showed rAAV's ability to transport small interfering RNAs for therapy of disease (Tomar et al., 2003). Serotype AAV5 is specific to the lung and retina, but the horse has neutralizing antibodies that prevent effective transfection in that model. Serotype AAV6 is specific to the heart and lung tissues and AAV7 to the retina. Serotype AAV8 is another serotype that is effective in crossing blood barriers and has capsids specific to the liver, retina, pancreas, and heart. Finally, the AAV9 serotype is very broad in its capabilities of transduction in many tissues and cells. Its capsids are specific to the liver, heart, brain, lungs, pancreas, and kidney. It will also remain in the animal's circulation at a prolonged rate compared to the other serotypes (Mason et al., 2015).

Transfection in orthopedic projects proves more difficult than in vital, soft tissue-based organs of the body. However, transfection of tissues or cells near or adjacent to the target tissue has been shown to also create positive outcomes in overall delivery goals.

### **CHAPTER III**

## **MATERIALS AND METHODS**

### **Ovariectomy**

All surgical procedures were conducted at the USDA ARS Poisonous Plant Research Laboratory under the approval of the USU IACUC #10046. Removal of ovaries in the seven mature, Suffolk-cross ewes was accomplished using a common ovariectomy procedure. Briefly, sheep were anesthetized using intravenous ketamine (3mg/kg IV, MWI Animal Health, Boise, ID) and diazepam (0.5–1mg/kg IV, MWI Animal Health, Boise, ID) followed by intubation and maintained using 1-2% isoflurane in oxygen. Using standard sterile surgical techniques, the skin and abdominal musculature were incised at the site of the lower abdomen, ovaries were removed and the subcutaneous tissue was closed using 2-0 Vicryl (Ethicon, Cincinnati, OH) with a continuous pattern and the final layer was closed using 3-0 Vicryl (Ethicon, Cincinnati, OH) on the subcuticular tissues to produce a buried suture closure of the skin, also using a simple continuous pattern.

### **Cranial Cruciate Ligament Desmotomy**

Surgical destabilization of the left stifle in the ewes was accomplished using a CCLD on eight sheep. Briefly, sheep were anesthetized using intravenous ketamine (3mg/kg IV, MWI Animal Health, Boise, ID) and diazepam (0.5–1mg/kg IV, MWI Animal Health, Boise, ID) followed by intubation and maintained using 1-2% isoflurane

in oxygen. Using standard sterile surgical techniques, an arthrotomy dorsomedial to the medial collateral ligament was performed and the anterior tibial attachment of the medial meniscus and anterior cruciate ligament was identified; forceps were passed behind the CCL to isolate this structure, which was transected with a scalpel blade. The effectiveness of the desmotomy was confirmed based on the increased anterior drawer sign. Hemostasis was ensured using electrocautery where necessary. Before closure, the joint was lavaged with sterile saline. The joint capsule and associated connective tissue were closed using a simple interrupted pattern using 1 Vicryl (Ethicon, Cincinnati, OH). The subcutaneous tissue was closed using 2-0 Vicryl (Ethicon, Cincinnati, OH) on a continuous pattern and the final layer was closed using 3-0 Vicryl (Ethicon, Cincinnati, OH) on the subcuticular tissues to produce a buried suture closure of the skin, also using a simple continuous pattern. Aluminum bandage spray was applied to the wounds once post-operatively (AluSpray, Neogen Animal Safety, Lexington, KY).

All animals received cephalosporin antibiotics (West-Ward Pharmaceuticals Corp, West Eatontown, NJ) preoperatively and analgesics (fentanyl patch, 100 ug/hr [Mylan, Canonsburg, PA] and meloxicam, 1 mg/kg IV [MWI Animal Health, Boise, ID]) for 48 hr postoperatively. Following surgical recovery, all animals were group-housed in small (8 ft × 10 ft) indoor pens. Three days postoperatively, the animals were moved to group housing in 10 ft × 10 ft indoor pens.

### **Treadmill Training**

All 15 subjects were allowed to recover from surgery for at least two weeks to

ensure full healing of surgery sites. Subjects were then habituated to being halter tied and led. Then subjects were introduced to the treadmill machine by being led up and down the ramp. When the subjects were accustomed to the treadmill, they were then tied to the front gate and speed was very gradually increased over several training sessions. Once sheep were familiarized with the treadmill motion, the oblique angle gates were incorporated, and trial runs were completed before the official exercise regimen was started to acclimate the animals to the procedure and ensure proper walking and safety.

### **Viral Injections**

Three subjects from each surgery group were randomly selected to receive the *TIMP-3* transgene delivered via an adeno-associated viral vector directly into the left stifle joint. Sheep were restrained on their backs for better access to each stifle joint. The left stifle was fully flexed and then pulled back slightly to open the joint for better access to the injection site. A 21-gauge needle was attached to a 6mL syringe to load  $3.42 \times 10^{12}$  CC of the *TIMP-3* transgene in 100  $\mu$ l of sterile saline into the syringe. Wool was sheared and skin was disinfected in a 4-inch radius around injection sites. A 23-gauge  $\frac{3}{4}$  inch butterfly catheter was placed in the lateral and medial openings of the joint. The syringe was attached to the butterfly catheter and expelled into each joint capsule. Injections were given only in the left stifles, but in the medial and lateral compartments of the stifle for a total of  $6.84 \times 10^{12}$  CC per joint. The joint was flexed several times to ensure the dispersal of the solution within the three, communicating joint capsules of the stifle joint.

### **Radiograph Collection**

Radiographic images were collected for all 15 subjects at four time points throughout the study. Each collection consisted of four different views of the stifle for both right and left joints. All personnel wore proper PPE including lead aprons, gloves, and thyroid shields. One person restrained the front of the sheep, another held the plate and limb of the sheep in place for the desired radiograph, another positioned the radiograph machine at the desired angle for radiograph and one person recorded notes and adjusted settings on the radiograph machine's computer.

Both left and right stifles were radiographed at four different angles. The first was a caudal-cranial view requiring the plate cranial of the stifle and the radiograph machine caudal of the stifle. The second was a medial-oblique view which required the plate medial-oblique to the stifle and the radiograph machine lateral-oblique to the stifle. The third was a lateral-medial view requiring the plate medial to the stifle and the radiograph machine lateral to the stifle. The fourth was a skyline flexed view which required the handler to flex the stifle joint completely and hold the plate beneath the flexed limb and the radiograph machine directly above the flexed stifle joint. This final view allowed for the opening of the joint space in the stifle.

### **Oblique-Angle Exercise**

All 15 subjects were walked for 32 minutes, every other day for 16 weeks at 80 meters/minute on an equine treadmill (Horse Gym USA, LLC, Wellington, Florida) at a right oblique angle. Custom-made USU Oblique Angle Exercise Gates were attached to

the treadmill (Hill et al., 2017). Subjects were led onto the treadmill individually and halter-tied and monitored at the front of the treadmill to force constant walking. The gates were then locked into place at an oblique angle. The treadmill was set for 32 minutes and speed began low and was slowly increased within 1-2 minutes to 80 meters/minute (a fast-paced walk). Subject gait was closely monitored to ensure the proper angle of exercise. Any animal that faltered or fell was immediately released. Gait was measured in collaboration with members of the USU Kinesiology and Health Science department.

### **Urine Collection**

Urine was collected at eleven time points throughout the study for the measure of specific and total protein. One of two methods was used to collect urine. The first method involved a collection cup tethered to a long pole that was held behind the subject as they walked on the treadmill for a free catch collection. The second method involved haltering and tying to a secure pole. The subject was restrained by one or two handlers, one of which manually stimulated urination. A third person performed a free catch collection with a collection cup and transferred to sterile 15mL tubes. Urine was cooled in Styrofoam coolers with ice and aliquoted into Eppendorf tubes. Samples were stored in 1.5mL aliquots at -80° until use in further analysis.

### **Serum Collection**

Blood was also collected at 11 time points throughout the study, starting the day of surgery and every 3 weeks after until collection. The blood samples were then spun



down to isolate serum. Blood was collected into Vacutainers (Becton-Dickinson, Franklin Lakes, NJ, USA) tubes with anticoagulants (red top tubes) via jugular venipuncture. After collection, the vein was held off for at least 60 seconds to prevent hematoma formation. After being allowed to clot for 30 minutes at RT, blood samples were centrifuged at 2000 RPM for 30 minutes until the serum was adequately separated. The serum was pipetted into aliquots of 1 mL each in Eppendorf tubes. Samples were stored at -80° until use in further analysis.

### **Gait Data Collection**

Gait analysis was performed at week 16. Subjects were trained to walk at an oblique angle during forced exercise; however, during gait analysis data collection, they were walked straight. A digital video camera was positioned alongside (approximately one meter from the right flank) the treadmill and used to record bilateral hind limb stance time. The sampling duration for each subject was a minimum of two minutes with the video camera sampling at 120 Hz. The video was imported into Kinovea (Kinovea: open source project, [www.kinovea.org](http://www.kinovea.org)) for digitization and manual data extraction. The hind hooves of the sheep were painted with bright paint to increase the contrast between the limb and treadmill to help aide with the digitization process. Stance time in seconds was extracted from the video of the left and right limb from 20 gait cycles.

### **Necropsy**

After completion of the 16-week exercise regimen, sheep were transported to the

Utah Veterinary Diagnostic Laboratory (UVDL) from the Poisonous Plants Research Laboratory (PPRL). Animals were restrained and a veterinarian humanely euthanized each in accordance with the IACUC protocol with an overdose of  $\geq 100$  mg/kg pentobarbital/phenytoin administered intravenously. After the declaration of death by the veterinarian, sheep were moved to the necropsy floor.

### **Whole-Brain Collection**

Subjects were positioned ventrally position and the hide was removed in order to allow a saw to access the skull. Two parallel, transverse incisions were made on the top center of the skull. A third incision was made on one of either side of the head to create a flap in the skull. A flathead screwdriver was used to wedge into the skull and use leverage to remove the top and cut out a small section of the brain.

### **Organ Samples Collection**

Sheep were laid in lateral recumbency. A necropsy knife was used to reflect the ventral and lateral skin on the abdomen of the sheep. An incision in the center of the ventral abdomen was made and spread from the lower abdomen to the base of the ribcage. Small sections of the liver, spleen, and kidney were located and retrieved. The ovaries were located in those sheep who still had them and retrieved. An incision was made in the diaphragm at the top of the abdominal cavity to retrieve the lung from the thoracic cavity.

### **Popliteal Lymph Node Collection**

The popliteal lymph node was located in the outer, caudal portion of the sheep's

flank. To retrieve this removal of the hide from the point of the hip to the tarsus was accomplished. From the lateral side, the distal attachment of the gluteobiceps muscle was identified. The distal tendinous attachment of the gluteobiceps to the stifle was dissected. The gluteobiceps were identified and the fatty connective tissue bundle superficial and caudal to the gastrocnemius was removed. The fatty connective tissue bundle was palpated for a small, dense lymph node. The lymph node was isolated and removed from the fatty connective tissue bundle.

### **Stifle Injection, Dissection, and Retrieval**

Sheep stifles were needed for micro-CT analysis of cartilage and bone deformation. The entire stifle was skinned from the cannon bone to just below the pelvic bone. Once skinned, a 30mL syringe and 14-gauge needle were used to draw up 30mL of 10% Formalin. A butterfly catheter was placed in the opening of the joint. The stifle was fully flexed and then pull back slightly to open the joint. A 30mL syringe of 10% Formalin was attached to the butterfly catheter and expelled into the joint capsule. This was repeated for both stifles. Once both joints were injected with formalin, stifles were collected. All tendons, ligaments, and muscles were cut away from the tibia and femur surrounding the joint capsule. The joint capsule was kept intact. Once all soft tissues were cut away, a saw was used to cut the tibia just below the tibial crest. The saw was also used to cut above the joint capsule on the femur no higher than an inch to remove the stifle from the carcass. Once removed, stifled were notched using a bone file on the cranial portion of the tibial crest for identification. Stifles were then submerged in formalin for fixation.

### **Bone Marrow Collection**

Bone marrow was collected from the distal femur using the bone curette to scoop the marrow from the cut portion.

### **Urine DMMB Analysis**

A modified DMMB assay was used on extracted urine samples to determine GAG concentration as a marker of cartilage matrix degradation. The extraction of GAG from urine samples included adding 100  $\mu$ l HCl to 1.0 mL of urine to pH 6.0. Subsequently, 25.5  $\mu$ l of cetyltrimethylammonium bromide (50 g/L) was added to the samples (17  $\mu$ l /ml) and the samples were stored at 4°C for 24 hr. Samples were then centrifuged at 10,000*g* for 60 min at 4°C; the supernatant was removed and the pellet was washed with 95% ethanol and again stored at 4°C for 24hr. The samples were then spun at 5000*g* for 30 min at room temperature, the supernatant was removed and the pellet was dried at 60°C and after the addition of 150  $\mu$ l M NaCl, briefly vortexed, and centrifuged at 10,000*g* for 30min at 4°C. The supernatant was then removed, and the pellet discarded, and 95% ethanol was added to the supernatant (750  $\mu$ l), which was briefly vortexed and stored at -20°C overnight. Samples were then centrifuged at 5,000*g* for 30 min at room temperature and the supernatant removed. The pellet was dried at 60°C. Next, 75  $\mu$ l deionized water was added to the dried samples, mixed the samples well and lyophilized in a speed vacuum concentrator for 45 min. Then, 150  $\mu$ l of acetone was added and samples were stored at -20°C for 24 hr. Samples were then centrifuged at 5,000*g* for 15 min at room temperature, the supernatant was removed and the pellet was then dried at

60°C, 15 µl of deionized water was added and these samples were used in the DMMB assay. The absorbance of the samples was determined at 525 nm, normalized to 690 nm, and plotted against a chondroitin sulfate-6 (CS) standard curve between 100 and 1000 µg/ml CS.

### **Serum Analysis (DMMB)**

A modified DMMB assay was used on extracted serum samples to determine GAG concentration as a marker of cartilage matrix degradation as well. Serum was diluted 1:1 with PBS medium, then 1:1 with digestion buffer and incubated at 65° C for 30 minutes. These were then used in the DMMB assay. The absorbance of the samples was determined at 525 nm, normalized to 690 nm, and plotted against a chondroitin sulfate-6 (CS) standard curve between 100 and 1000 µg/ml CS.

### **Serum Analysis (ELISA)**

Concentrations of the aggrecan epitope CS846 were measured using a commercial enzyme-linked immunosorbent assay kit (ELISA, IBEX Diagnostics, Montreal, Quebec, Canada) as a marker of aggrecan synthesis (and degradation).

### **Protein Analysis (BCA)**

A Bicinchoninic Acid assay was used for the colorimetric detection and quantitation of total protein in the serum and urine for the standardized measurement of total GAG and specifically aggrecan measurements in these biomarkers. Samples were

thawed and diluted 1:10 (urine) and 1:100 (serum) for use in the BCA assay. The absorbance of samples was determined at 562 nm, normalized to 690 nm.

### **Radiograph Analysis**

Radiographic analysis was completed by three blinded graders over 2 days to reduce variability. All 480 radiographs were viewed using the eFilm Workstation (IBM Watson Health, Chicago, Illinois, 60602). One of these graders was a veterinarian. The grading system used was developed in the pilot study (Hill et al., 2017) and is similar to the Kellgren-Lawrence grading scale. Osteophytosis was graded 0-4 in severity and the location of abnormality was noted.

In addition to osteophytosis, osteopenia and sclerosis were also measured, however, they were not prevalent in this study. Measures of joint space were taken from the caudal-cranial view along the tibial plateau using the eFilm Workstation (IBM Watson Health, Chicago, Illinois, 60602) software tools. Measurements of the lateral and medial condyle were recorded, and joint space measurements were taken at 40% of the lateral condyle proximal from the lateral side and at 50% of the medial condyle proximal from the medial side for every joint image.

### **Statistical Analysis**

The following statistical analysis was completed for the ELISA, DMMB measurement completed using serum and DMMB measurement using urine. A probability of  $p < 0.05$  was considered significant. The area under the curve of each

individual was calculated from 77 days post-surgery to collection. A two-way ANOVA (GraphPad Software, Inc., La Jolla, CA, 92037) was completed with the inclusion of multiple comparisons assessing the averages of each area under the curve calculation. This was done to note the similarity or dissimilarity between treatment groups. These results did not reach statistical significance.

Statistical analysis was completed for the gait datasets. A probability if  $P < 0.05$  was considered significant. The total average of stance time from each individual, using 20 gait cycles from separate left and right hind limbs at a single time point near the end of the exercise regimen, was measured. A two-way ANOVA (GraphPad Software, Inc., La Jolla, CA, 92037) was completed with the inclusion of multiple comparisons assessing the averages of each measurement. This was done to note the similarity or dissimilarity between treatment groups as well as between left and right hind limbs. These results did not reach statistical significance.

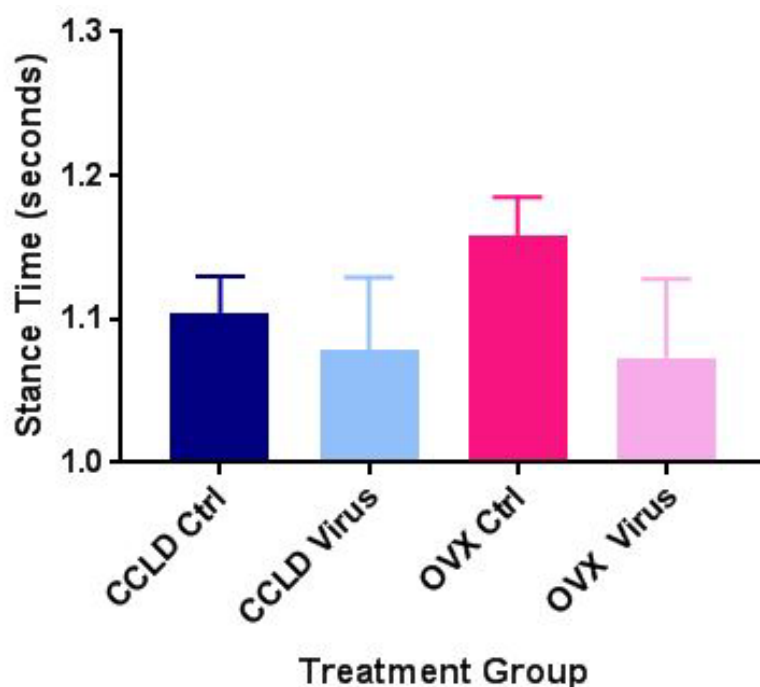
Statistical analysis was completed for the radiographic datasets including osteophytosis measurements, joint space measurements, and joint width measurements. A probability if  $p < 0.05$  was considered significant. The total average osteophytosis score of each individual, from four time points beginning before the exercise regimen up to just before collection, was measured. A two-way ANOVA (GraphPad Software, Inc., La Jolla, CA, 92037) was completed with the inclusion of multiple comparisons assessing the averages of each measurement. This was done to note the similarity or dissimilarity between treatment groups. These results did not reach statistical significance.

## CHAPTER IV

### RESULTS

#### Gait Analysis

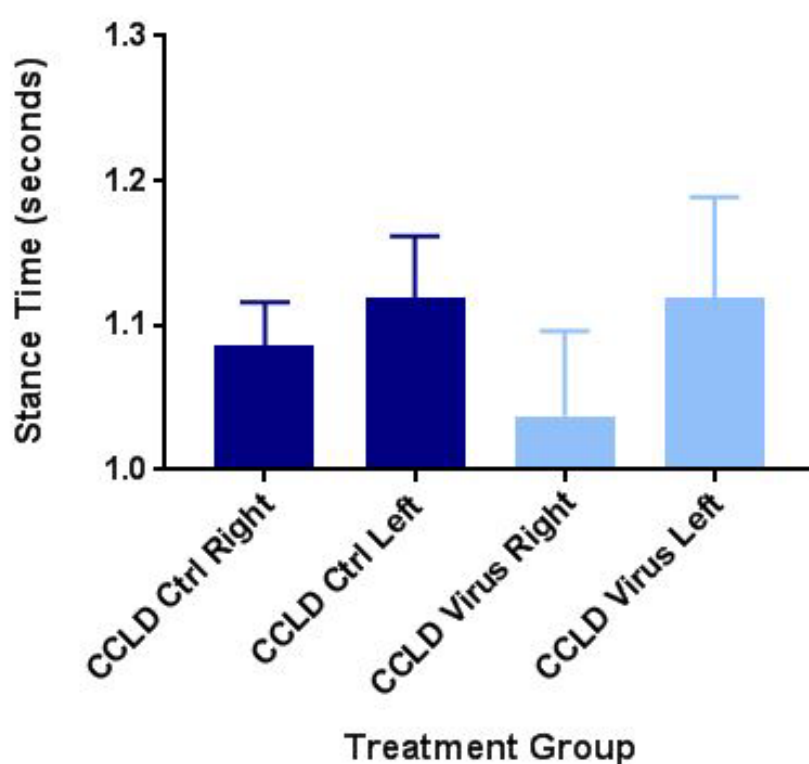
Analysis of gait was recorded during the 16<sup>th</sup> week of exercise. Twenty normal gait cycles were recorded, and stance time was measured in seconds. An average increase in stance time for control groups compared with their respective treatment groups was observed (Figure 9). There was a numerical increase in average stance time for the OVX control group compared with the CCLD control group. A numerical increase in average stance time for control groups compared with their respective virus groups was observed. There was no difference in the average stance time among virus groups (Figure 9).



*Figure 9.* Comparison of average stance time in both left and right limbs for all four experimental groups. Error bars represent standard error of the mean.



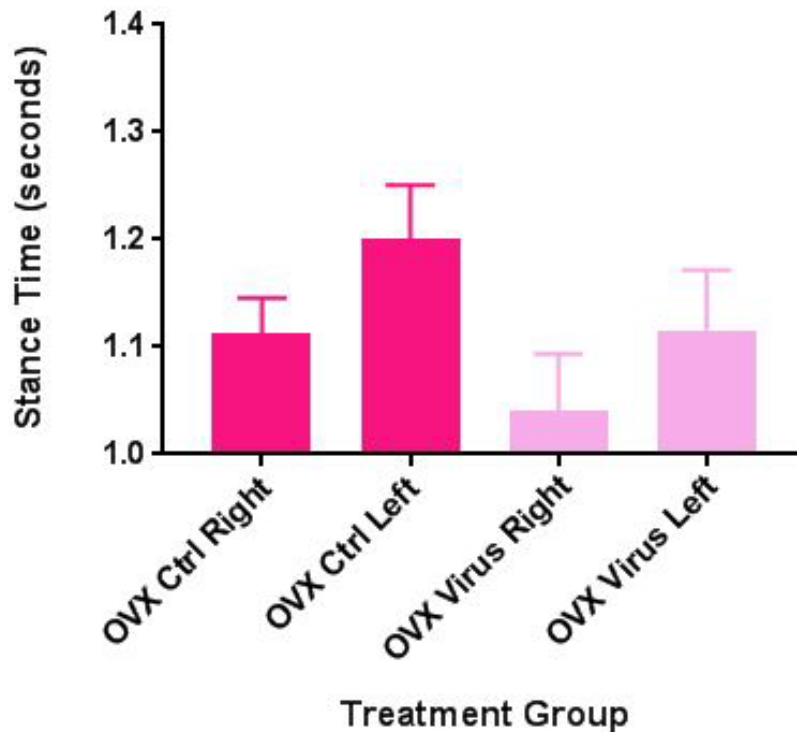
There was a numerical average increase in stance time for left limbs compared with their contralateral right limbs. No difference in stance time for left limbs in either CCLD control or virus groups was recorded. There was a numerical increase in average stance time in CCLD control right limbs compared with CCLD virus right limbs (Figure 10).



*Figure 10.* Comparison of average stance time for separate left and right limbs within the CCLD experimental groups. Error bars represent standard error of the mean.

A numerical increase in stance time for OVX control left limbs compared with OVX control right limbs was observed as well as an average numerical increase in stance time for OVX virus left limbs compared with OVX virus right limbs. There was a numerical increase in average stance time for OVX control left limbs compared with

OVX virus left limbs. A numerical increase in average stance time for OVX control right limbs compared with OVX virus right limbs was recorded (Figure 11).



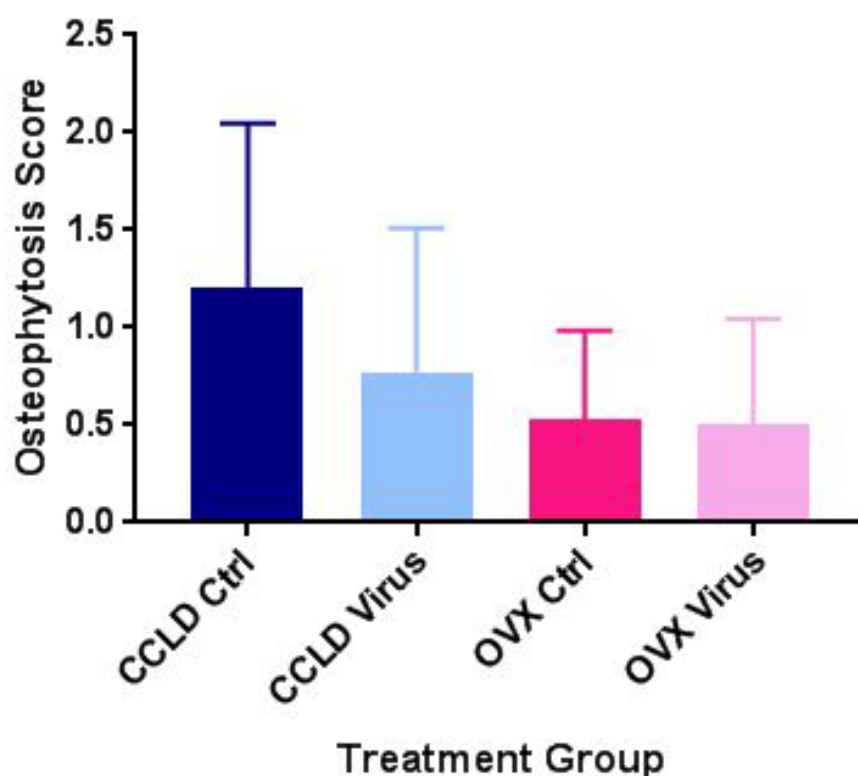
*Figure 11.* Comparison of average stance time for separate left and right limbs within the OVX experimental groups. Error bars represent standard error of the mean.

### **Radiographic Analysis**

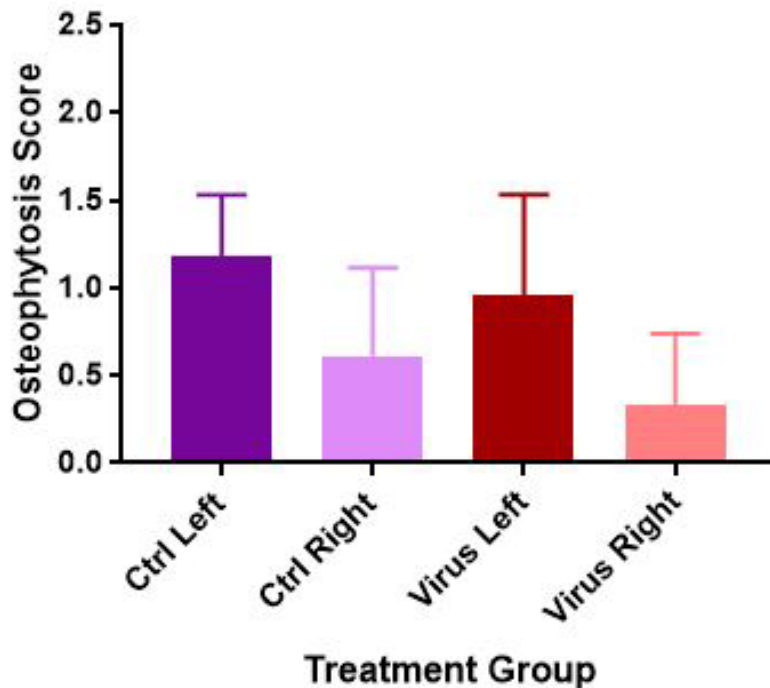
Radiographic data were collected at four time points, one just prior to the start of running and three more during the exercise routine. Four views of both left and right limbs on each sheep were recorded: a caudal-cranial view, a medial-oblique view, a lateral-medial view, and a flexed skyline view.

## Osteophytosis

There was a numerical increase in mean osteophytosis score for the CCLD control group compared with the CCLD virus group. No statistical difference was seen in the average osteophytosis score for OVX experimental groups (Figure 12). There was a numerical increase in average osteophytosis scores for left limbs were increased compared with right limbs in both control and virus experimental groups. Osteophytosis of the left limbs of the combined control groups was numerically greater than the right limbs of the combined virus experimental groups (Figure 13).



*Figure 12.* Comparison of average osteophytosis score in both left and right limbs for all four experimental groups. Error bars represent standard error of the mean.



*Figure 13.* Comparison of combined control and virus group's average osteophytosis score by limb. Error bars represent standard error of the mean.

### **Joint Width**

The width of the tibia and femur were measured on each caudal-cranial view of the radiograph to quantify joint widening over time in correlation with abnormal bone growth or osteophytosis. A numerical increase in the difference between control groups compared with their respective virus groups was observed (Figures 14 and 15).

### **Joint Space**

The joint space was measured between the lateral and medial condyles of the femur on each caudal-cranial view of the radiograph to quantify joint space narrowing over time in correlation with the progression of OA and degradation of cartilage. There was numerically increased narrowing in the CCLD control group compared to a widening

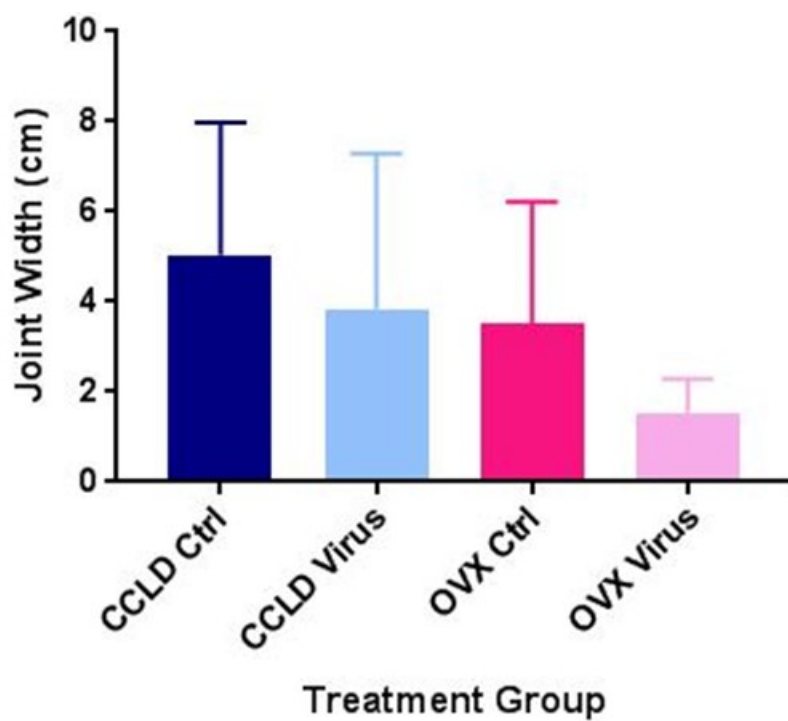


Figure 14. Comparison of average femoral change in width from the first radiograph to the last radiograph for all four groups. Error bars represent standard error of the mean.

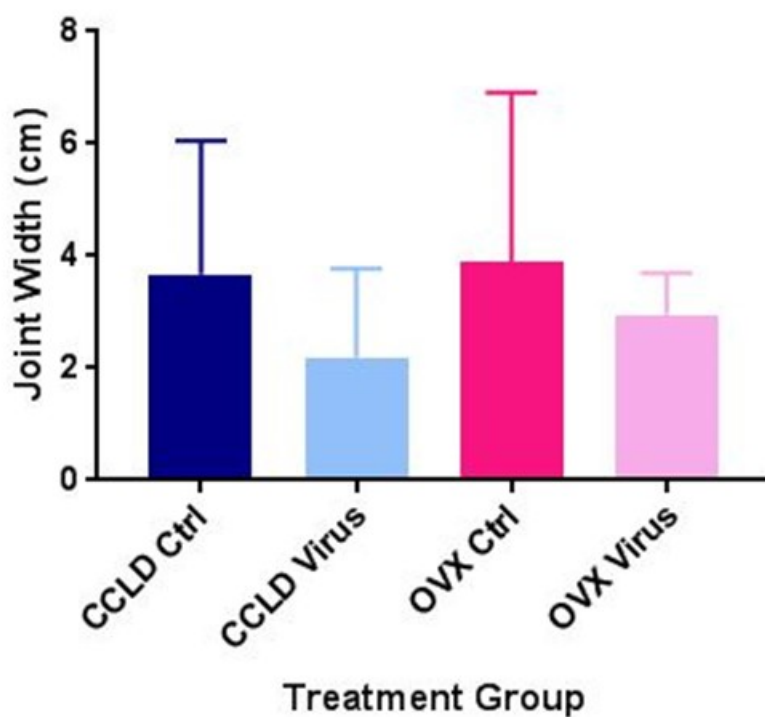
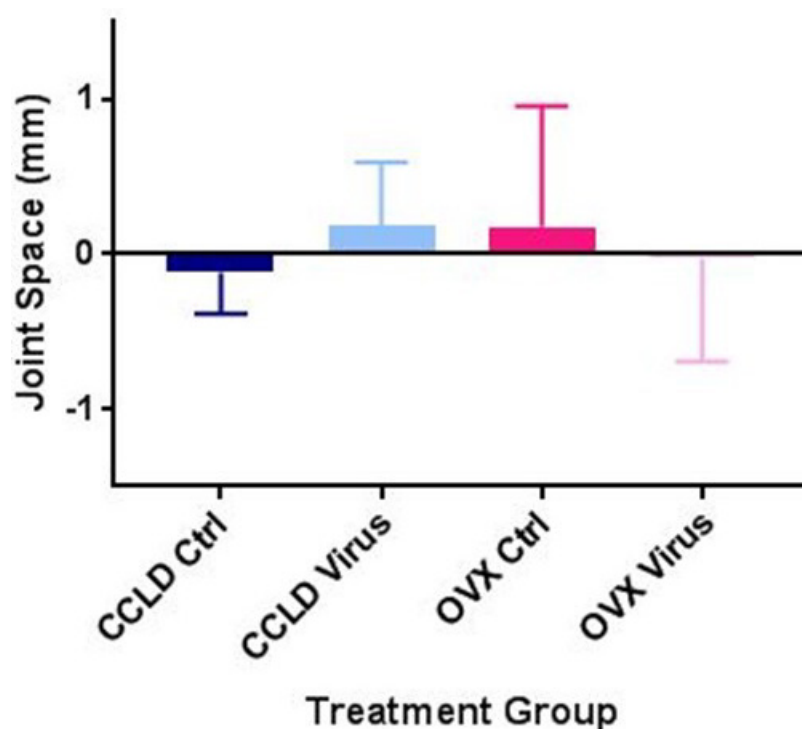


Figure 15. Comparison of average tibial change in width from the first radiograph to the last radiograph for all four groups. Error bars represent standard error of the mean.

in the CCLD virus group. There was numerical joint space widening in the OVX control group compared to narrowing in the OVX virus group (Figure 16).



*Figure 16.* Comparison of average joint space change of both limbs from first radiograph to last radiograph for all four groups. Error bars represent standard error of the mean.

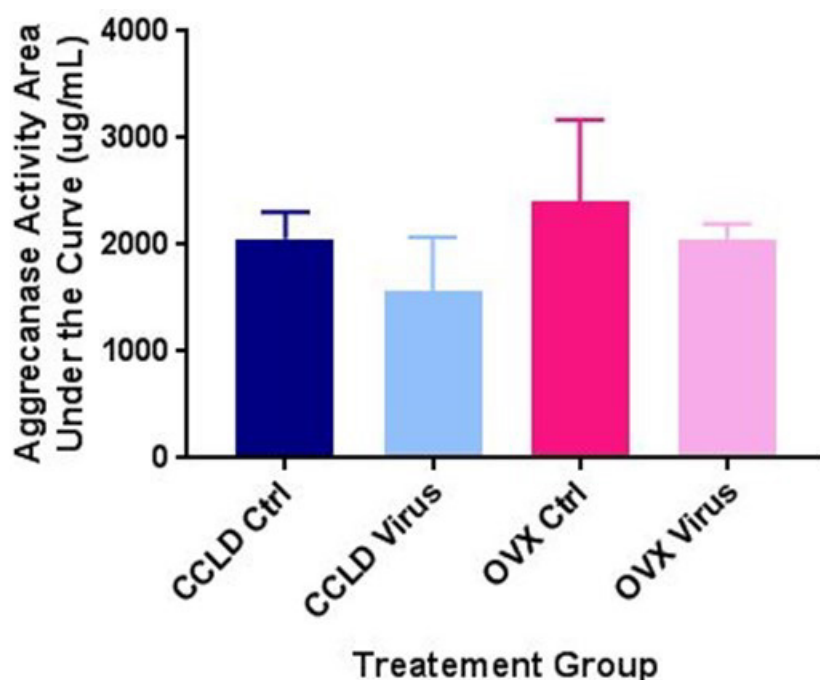
### Biomarker Analysis

Biomarkers were collected at 11 time points throughout the study including urine and blood. Urine was aliquoted and frozen at  $-80^{\circ}$  until the assay was conducted. Blood was processed and serum was frozen at  $-80^{\circ}$  until assays were conducted.

### Aggrecanase Activity

There was a numerical increase in area under the curve of aggrecanase activity for

control groups compared with their respective virus groups. A numerical increase in area under the curve for the OVX control group compared with the OVX virus group was recorded. There was a numerical increase in area under the curve aggrecanase activity for the OVX control compared with the CCLD control group. A numerical increase in area under the curve aggrecanase activity for the OVX virus compared with the CCLD virus group was observed (Figure 17).

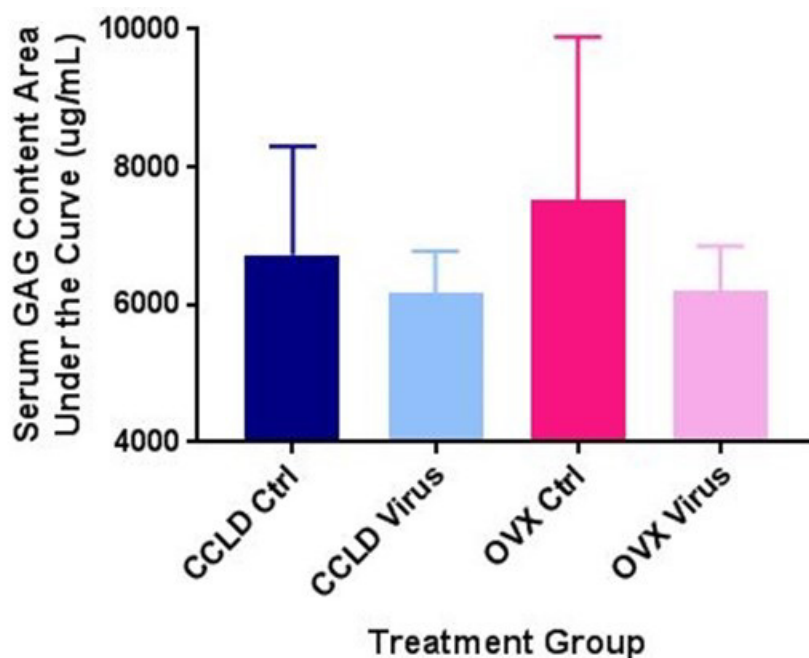


*Figure 17.* Comparison of area under the curve of aggrecanase activity for all four experimental groups. Error bars represent standard error of the mean.

### **Serum Glycosaminoglycan Content**

Serum samples were collected throughout the study and analyzed using a modified DMMB Assay to quantify glycosaminoglycan content over time. There was a numerical increase in area under the curve of serum glycosaminoglycan content for control groups compared with their respective virus groups. There was no difference in

the average area under the curve for serum glycosaminoglycan content between CCLD and OVX virus groups (Figure 18).

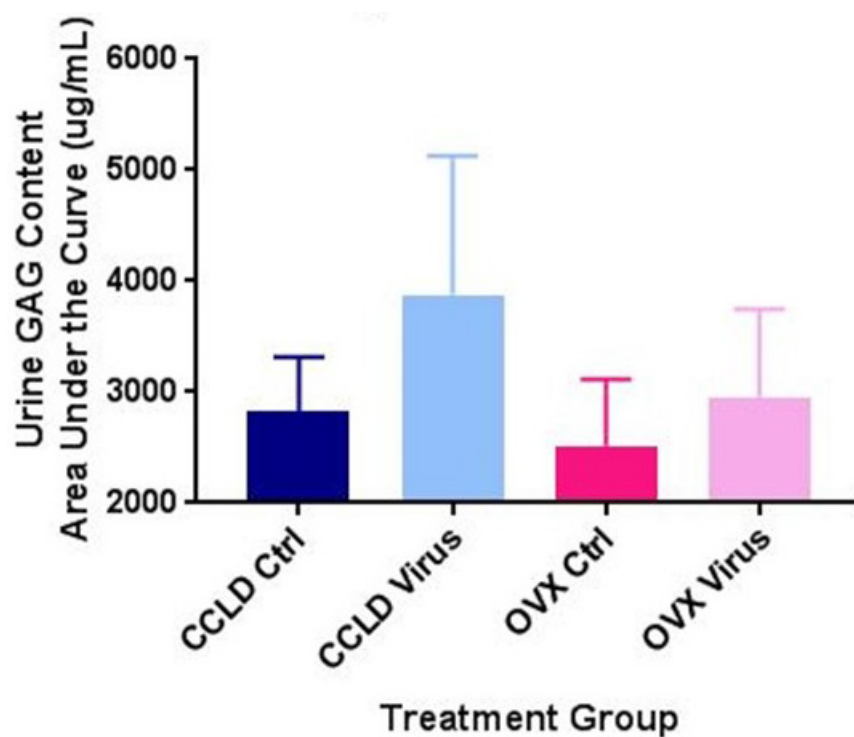


*Figure 18.* Comparison of area under the curve of serum glycosaminoglycan content for all four experimental groups. Error bars represent standard error of the mean.

### Urine Glycosaminoglycan Content

Urine samples were collected throughout the study and analyzed using a modified DMMB Assay to quantify glycosaminoglycan content over time. A numerical increase in average area under the curve urine glycosaminoglycan content for virus groups compared with their respective controls was observed. There was a numerical increase in average area under the curve urine glycosaminoglycan content for the CCLD virus group compared with OVX virus groups and CCLD control group compared with the OVX control group (Figure 19).





*Figure 19.* Comparison of area under the curve of urine glycosaminoglycan content for all four experimental groups. Error bars represent standard error of the mean.

## CHAPTER V

### DISCUSSION

In the current study, we used CCLD and OVX surgeries, coupled with oblique angle forced exercise to induce OA in an ovine model. Sheep receiving TIMP-3 gene therapy prior to the exercise regimen had reduced OA lesions and biomarkers of OA. There was a numerical increase in abnormal bone growth, a numerical decrease in biomarkers of cartilage degradation and a mixed result in gait improvement when comparing the untreated and treated groups. In a 2015 study by Hill et al., a CCLD was performed on mature, Suffolk-crossed ewes as well as a similar oblique-angle forced exercise routine and was successful in producing clinical signs of OA. The study laid the foundation for the current research: furthering the investigation of a preventative gene therapy. Ovariectomy has been shown to increase clinical signs of OA (Sniekers et al., 2008). The multiple measurements evaluated in this study suggest an overall improved outcome for subjects who received viral injections.

The effect of OA on stance time has been reported in multiple studies (Fang et al., 2011; Ornetti et al., 2011; Watanabe, Shimada, Sato, Tsutsumi, & Sato, 1998; Watanabe, Shimada, Kagaya, & Sato, 1999; Watelain, Dujardin, Babier, Dubois, & Allard, 2001) showing an increase in stance time is positively correlated with OA progression. Stance time is a measurement of the length of time the limb is in stance phase. Stance phase involves the foot or hoof touching the ground during gait. It is hypothesized that stance time reflects a decrease in stability in limbs affected by the disease. Our results display a numerical increase in stance time for our control groups compared to our virus groups.

When looking at just CCLD groups, the left stifle had numerically increased stance time compared to the right in both treated and untreated groups. As this was the limb where transections were performed and the limb forced into an abnormal posture during exercise, these results reflect what we expected to see. When looking at just OVX groups, there was also a numerical increase in stance time for left limbs compared with the right limbs. This further supports the effectiveness of oblique-angle forced exercise to exacerbate the destabilizing effects of OA. These gait measurements were only recorded at one time point near the end of exercise, so changes over time could not be evaluated.

Average osteophytosis was numerically greater in the CCLD control group compared to the CCLD virus group. This reflects similarly the results presented by Hill et al. (2017) where osteophytosis increased in sheep who underwent surgery and exercise and is further supported by (Mason et al., 2017) that indicates a decrease in osteophytosis in rat stifles that were transduced with the *DKK-1* transgene. DKK-1 is a known inhibitor of the Wnt pathway which drives bone remodeling (Amrein et al., 2014). There was no difference between average osteophytosis scores for OVX virus and OVX control groups. The sheep who underwent an ovariectomy finished the study just seven months later and the short timespan could have prevented a greater impact on bone growth from lack of hormonal influence (OVX effects) to occur. In future studies, allowing sheep who undergo ovariectomy more time post-operatively could produce a greater difference in abnormal bone growth.

There was a numerical increase in joint width over time in all treatment groups, but a greater change in control groups. Joint width positively associated with

osteophytosis in CCLD groups, as there was a greater numerical increase in width for control groups compared to virus groups, comparable with the results from Hill et al. (2017). In OVX groups, there were trends for numerically increased joint width as well in control groups compared to virus groups. There was no change in joint space. There was variability in radiograph positioning and sheep positioning as is expected in any live animal study. Therefore, radiographic measures of joint width and space should be interpreted cautiously.

Mason et al. (2013) studied the *TIMP-3* transgene in an *in vitro* setting to determine the effects that up-regulation had on cartilage. A co-culture of cartilage biopsies displayed a decrease in aggrecanase activity and an increase in retained GAG in those samples cultured with the TIMP-3 protein, especially those cultured with the rAAV-*TIMP-3* transduced cells. These results can be compared with what we found in our biomarker serum aggrecanase activity and serum GAG results. They do not, however, support our urine GAG results and this could be explained by assay variability and sensitivity. We showed an increase in aggrecanase activity for Control groups compared to virus groups overall and to a larger scale in the OVX groups. This could be a result of the decrease in hormonal interactions to preserve cartilage in those sheep without ovaries. Aggrecan is a large, aggregating proteoglycan that is one of the first to be degraded from cartilage during OA. Frisbie, Ionescu, Poole, Chapman, and McIlwraith (1999) measured the serum aggrecan content of horses with joint disease with a positive correlation. Degeneration of cartilage is correlated with an increased appearance of GAG in serum and urine. A study conducted by Frisbie et al. in 2008 showed an increase in serum GAG

in OA-affected horses both with and without exercise compared to horses with no OA.

Overall, our results displayed a numerically greater impact on clinical signs of OA in bone in our CCLD groups compared to our OVX groups. This could be explained by the decreased hormonal effect in OVX sheep. We also saw a similar degradation of biomarkers overall in both groups, but a numerical increase in controls compared to their respective virus groups indicating a possible negative effect on osteoarthritic changes with gene therapy.

In conclusion, the current research demonstrates a numerically positive association for the use of the *TIMP-3* transgene for the negative effects of osteoarthritis. Further research is still needed to prove its effectiveness in the form of an increased sample size, prolonged and a greater degree of intensity in an exercise regimen, and further developed methods for radiographic image acquisition on a live animal model. Additional studies with these factors in mind could provide a more significant divergence between subjects receiving and not receiving gene therapy. This TIMP-3 gene therapy appeared to decrease or slow down the development of OA and might decrease unnecessary suffering for millions of patients.

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