ADVANCING METHODS FOR MULTISCALE MECHANICS IN ORTHOPAEDIC RESEARCH

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY UNIVERSITY OF FLORIDA 2017
To my family
ACKNOWLEDGMENTS

First and foremost, I thank my supervisor and chair, Dr. Kyle Allen, for his guidance over four years of research, statistics, writing, and life planning. I will always be grateful for your last-minute phone call saying you found money to hire me as a graduate student. Now that I know the ins-and-outs of funding, I am more thankful than ever that you worked hard and found a way for me. Thank you. I also thank members of my supervisory committee for their support: Scott Banks, Peter McFetridge, Stanley Kim, and Kevin Vincent. I have tremendous respect for each of you.

I also thank my lab mates for their immense support: Heidi Kloefkorn, Eric Rohrs, MiRa Jacobs, Yash Shah, Elena Yarmola, and Brittany Partain. I have enjoyed working with all of you, and am grateful I had you for the past four years. An extra thank you goes to MiRa for helping me survive the past two years. We worked extremely well when we merged into a single “super grad student”. I also thank my dedicated undergraduate students: Courtney Kline, Ania Lipat, Babatunde Balogun, Samantha Haus, Danny Xie, and Danelle Amsellam.

Of course, I owe a lot to my extremely supportive and loving parents, Beth and Jeff Lakes. From a young age, they have always accepted me for who I am, but made sure I knew that I could get out Connersville, Indiana and see the world. I am also extremely lucky in having my grandparents, Jack and Shirley Lakes and Bill and Kaye Branson, who have provided lifelong support and love. I couldn't have done it without you. Finally, I thank my husband, Matthew Mills, who guided me to pursue a doctorate degree and has made me a better person and engineer. I love you all.
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Like many medical oriented fields, orthopaedics has struggled in transferring technology between preclinical and clinical research and in understanding the intersection of mechanics and biology at the body, joint, tissue, and cellular levels. This dissertation aims to advance methods for multiscale mechanics in orthopaedic research by 1) evaluating tissue mechanics changes caused by hydration, cellular removal, and physical modification of the knee meniscus and 2) scaling modern gait technology for use in rodents to study behavioral, mechanical, and biological changes in two preclinical models of osteoarthritis.

1) Orthopaedic allografts, synthetic replacements, and tissue engineered constructs must balance biological viability and mechanical integrity, which interact on different levels. This challenge is made more difficult by the varying methods used to validate orthopaedic tissue engineered constructs. For example, meniscus tears are the most common knee injury in the United States, but a long-term replacement is not yet available, and potential replacements often face mixed results. Thus, this work
examines the viscoelastic properties of the knee meniscus in different testing scenarios and structural modifications.

2) Traditionally, technologies to assess the functional capabilities of an orthopaedic disease are created for human research, such as force plates and motion capture systems. However, to simultaneously study the mechanics and biology of diseases such as osteoarthritis, these technologies must also be developed for preclinical animals. Adapting these technologies for preclinical models not only helps bridge preclinical and clinical research, but also provides much needed quantitative measures of animal behavior and pain. Thus, this work improves methods to assess gait mechanics in rodents and studies rodent gait in two models of osteoarthritis.

Although the ability to study the interactions of biomechanics from the body down to the cell in a single model remains in the future, this dissertation serves to improve orthopaedic research by evaluating two areas where improvements are needed: tissue and gait mechanics in preclinical research.
CHAPTER 1
MULTISCALE BIOMECHANICS FOR ORTHOPAEDIC RESEARCH

Multiscale biomechanics is a vast field of interdisciplinary research seeking to understand how biomechanics principles relate from the body, to the joints, to the tissues, and finally down to the cells (Figure 1-1). The term multiscale mechanics refers to the combination of these research areas from the body-level to the cellular-level. Entire careers may be built around understanding multiscale mechanics principles in one tissue and at one level, and seeking to understand how that one level affects others.

### Multiscale Biomechanics

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Figure 1-1. Multiscale biomechanics addresses biomechanics at the body, joint, tissue, and cell levels.

Evaluating the interactions at each biological level is difficult to accomplish in either humans or a single preclinical model; thus, various areas of multiscale mechanics have evolved for each level. For example, body kinematics in humans are commonly studied with motion capture systems and high-speed video cameras. From this foundation, joint-level kinematics methods grew to include more accurate kinematic
recordings via fluoroscopic recordings\textsuperscript{1–5}. Human kinetics studies evolved to include multi-component force plates. These technologies allowed for ground reaction force measurements, which in turn allowed analytic models began to be created to translate ground reaction forces to joint-level forces with varying degrees of accuracy\textsuperscript{6,7}. Here, certain kinetic measures have begun to be related to orthopaedic pathologies in humans, such as the knee adduction moment associating with osteoarthritis symptoms and severity\textsuperscript{8–10}.

At the joint level, direct joint forces have been estimated using cadaver joints with strain gauges and pressure sensitive films\textsuperscript{11}. Joint forces have also recently been estimated using inverse dynamic models which incorporate ground reaction forces, muscle activations, and individual anatomy\textsuperscript{12–14}. There has also been a small number of patients with force recording knee implants, which has provided a lot of unique data to the field\textsuperscript{15,16}. However, these forces apply to the surgically altered and synthetically replaced joint.

It is at the joint level where human biomechanics tends to reach the limit of ethical research, as we cannot ethically implant strain gauges or force sensors into a healthy joint, test the \textit{in vivo} mechanical properties of tissue, extract tissue samples from a joint without causing injury, or change the cellular environment in healthy patients.

Preclinical models provide the opportunity to more ethically study orthopaedic biomechanics at the tissue and the cellular levels. For instance, mechanical testing machines have allowed for characterization of biological tissues, although the tissues and joints are typically excised from cadavers or preclinical large animals. Mechanical
testing is also used to validate preclinical therapies, such as a potential bone grafts and soft tissue or whole joint replacements. Additionally, a multitude of biochemistry assays are readily available for preclinical animal models, allowing the biological responses to orthopaedic pathologies to be evaluated. Animal models also allow researchers to examine cartilage and bone remodeling via immunohistochemistry. Finally, animal models allow for alterations to the body to deduce what role certain orthopaedic tissues play in movement and rehabilitation.

Unfortunately, preclinical research is not currently able to study body-level kinematics due to being restricted to cadavers or small animals having excess skin and crouched walking positions. Additionally, preclinical research often cannot relate disease changes to pain and behavior, which is the main focus for human patients with joint diseases. As such, implants and other therapies may be tested in animal models, but it is difficult to quantify pain levels. Additionally, current methods used in preclinical research vary across both laboratories and species in terms of mechanical testing of biomaterials and tissue, pain assessment, and assessing mechanical function in animals. Thus, preclinical research tends to reach its limit when it comes to larger scale orthopaedic measures, while clinical research tends to reach limits as the scale gets smaller.

Given the respective limitations of clinical and preclinical research, a gap develops when either side has information and tools the other side needs (Figure 1-2, next page). Bridging this gap has been difficult due to cadavers not fully predicting the living body, cross species differences, and availability of research tools. Thus, the field of multiscale biomechanics remains segmented, and the full relationship between body,
joint, tissue, and cellular changes has been difficult to fully understand. To move the field forward, clinical and preclinical research must communicate across this gap to share technology, measurement strategies, findings, and ideas. By promoting the convergence of preclinical and clinical research, multiscale mechanics may eventually have means of incorporating all levels in a single research strategy.

![Figure 1-2. A “valley of death” between preclinical and clinical orthopaedic research.](image)

To be clear, the end goal of being able to study the interactions of biomechanics from the body down to the cell in a single model remains in the future. However, this dissertation serves to improve orthopaedic research by evaluating two areas where improvements are needed (Figure 1-3, page 18). Part 1 will examine tissue engineering, where researchers are working to create replacements for orthopaedic tissues. Here, balancing the biology and required mechanical durability for orthopaedic tissues has been difficult. As such, patients are still waiting on viable biologic replacements. For example, the knee meniscus plays a significant role in joint loading\textsuperscript{11,19–21}; however, the current standard of care is to perform a meniscectomy and remove the damaged portion of the meniscus. Therefore, numerous researchers have created options for meniscus replacement; however, validating these replacements has
proven difficult due to inconsistent testing across the field and differences in species. Thus, part 1 will evaluate *ex vivo* mechanical testing of the knee meniscus, taking into account tissue level changes caused by hydration, cellular removal, and physical modification of the tissue.

Part 2 will then examine quantitative measures in preclinical animal models of orthopaedic pathologies (Figure 1-3, next page). Preclinical animal work offers a relatively controlled manner to study disease progression in ways not possible in humans; however, outcome measures in preclinical research are often semi-quantitative at best. Therefore, quantitative gait analysis will be used to evaluate preclinical rodent models of osteoarthritis. Through the use of quantitative gait analysis, the current understanding of how OA pain and disability develop can be examined in rodent models, allowing researchers to evaluate changes in joint mechanics, inflammatory responses, neuropathy, or other biopsychosocial factors and their relationships to pain. Such research will define the fundamental principles underlying disease progression to improve orthopaedic research and treatment.

Overall, the work in this dissertation will advance the field of multiscale biomechanics toward a cohesive understanding of mechanical interactions throughout the body by critically evaluating the areas of tissue and gait mechanics in preclinical models. The following chapter beings part 1, with an introduction to the knee meniscus.
Figure 1-3: This dissertation will focus on two areas of multiscale mechanics. Part 1 will examine tissue mechanics of the knee meniscus, while part 2 will examine rodent gait in preclinical models of osteoarthritis.
Knee Meniscus Structure and Function

The knee menisci are semi-lunar soft tissues which lie on the medial and lateral aspects of the tibial plateau. On a macro-scale, the menisci appear white and collagen dense, with little vascularization. The circular shape of the lateral meniscus covers 80% of the lateral plateau, while the broader and less symmetric shape of the medial meniscus covers 60% of the medial plateau\textsuperscript{22,23}. In cross section, each meniscus is wedge shaped and conforms to the curvature of the femoral condyles to provide support. Each meniscus is not rigidly connected to the tibia, but has insertion points at the anterior and posterior horns. Thus, the meniscus has mobility to support the joint in various positions and deform to distribute load.

Each meniscus is made up of 70% water and 30% organic matter\textsuperscript{22,24}. The abundance of water creates drag forces as water is exuded from the tissue when load is applied, contributing to the viscoelastic properties of the meniscus. Of the organic matter, collagen makes up 75% of the dry weight\textsuperscript{25}. The outer periphery of the meniscus is primarily type I collagen, while the inner region is 60% type I and 40% type II collagen\textsuperscript{26,27}. The type I collagen in the inner region provides a majority of the mechanical strength of the meniscus due to the complex network of circumferentially aligned collagen fibers. Cells in the outer region are surrounded by type I collagen and are fibroblast-like, with oval fusiform shapes\textsuperscript{22,28}. Cells in the inner region are surrounded by collagen II, which is similar to articular cartilage; these cells are chondrocyte-like and sometimes referred to as fibrochondrocytes\textsuperscript{22,28}. Additionally, proteoglycans make up 2% dry weight of the meniscus and include aggrecan, decorin,
and biglycan\textsuperscript{29,30}. Aggrecan is a large molecule with chondroitin sulfate and keratan sulfate glycosaminoglycan chains; this combination creates a high charge density and hydrophobicity which resists compressive loading and gives the meniscus damping properties\textsuperscript{31–35}.

By the age of 9 months, each meniscus begins to lose vascularity until only the outer 10-30\% is vascularized by age 11\textsuperscript{23,36–38}. Because of this vascularization, injuries to the periphery of the meniscus may form clots and develop scar tissue across the tear; however, this scar tissue is much weaker than native tissue, leaving opportunity for secondary tearing\textsuperscript{22,39–41}. Injuries in the inner region of the meniscus are unable to repair, largely due to the lack of vascularization. Thus, these injuries continue to degenerate and ultimately cause pain and range of motion problems.

The outer third of the menisci and attachment horns are also innervated by parts of the posterior tibial nerve, obturator, and femoral nerves. As the knee is loaded, mechanoreceptors such as Ruffini endings, Pacinian corpuscles, and Golgi tendon organs send electrical signals to the outer nerves\textsuperscript{42–49}. These sensors likely play a role in the proprioceptive functions of the meniscus\textsuperscript{50,51}.

Each meniscus serves to transform high compressive body loads, up to 3.5 times body weight, into circumferential tensile loads along the circumference of the meniscus\textsuperscript{52,19}. This results in a distribution across the tibial plateau of 70-99\% of knee loads\textsuperscript{53}. Without the meniscus, stresses on the hyaline cartilage would be 2-3 times greater than normal\textsuperscript{37,54}. The menisci provide this mechanical function based on its complex structure of collagen fibers. On the surface, the meniscus has a random mesh of collagen fibers, while deep in the meniscus, circumferentially aligned collagen fibers
withstand tensile stresses and distribute load through the tissue\textsuperscript{27,37,55}. These circumferential fibers are held together by radial tie fibers. Additionally, large aggregating proteoglycans create a high fixed charge density and attract water into the meniscus, which further protect the tissue under compressive loading\textsuperscript{29,56}.

**Ex vivo Meniscus Testing and Mechanical Properties**

Like most biological tissues, the meniscus is a viscoelastic material, meaning it exhibits properties of a solid and a fluid depending on the rate of loading. Physiologically, the viscoelastic nature of the meniscus provides robust support for instantaneous loading and more relaxed support for long term loading, such as standing for long periods of time. Viscoelastic materials are difficult to test *ex vivo* and compare across laboratories due to different loading rates and mechanical testing set-ups. For instance, securing biological tissues for mechanical testing can be very difficult without causing damage; thus, laboratories have unique ways of gripping samples. Some methods include using sand paper between tension grips or securing compression samples with cyanoacrylate. Additionally, due to the viscoelasticity of the meniscus, *ex vivo* testing must incorporate sample hydration, which can be difficult in tension.

Physiologic measurements of meniscal stress and strain are difficult to quantify in living humans. Thus, estimates of meniscus loading are largely based on computational models and cadaveric studies. For tensile loading, finite element estimations report a 0.46 MPa circumferential stress in the meniscus\textsuperscript{57}. Jones et al. used strain gauges to measure circumferential strains and reported an average circumferential strain of 2.4±1.9\%\textsuperscript{58}. However, using finite element modelling, circumferential strains of only 0.23\% and radial strains of 0.26\% and 0.35\% were reported\textsuperscript{57,59}. In compression,
estimates of physiologic stress from finite element modelling are 0.24 MPa with physiologic strains of 1-10%\textsuperscript{37,57}.

Most common testing modes for the meniscus are tensile testing of the circumferential fibers and stress relaxation or creep testing in axial compression. For all testing, the strain rate is critical for viscoelastic materials. Strain rates for tensile testing of the meniscus include 0.1%/s, 0.5%/s, and 1.4%/s\textsuperscript{37,60–63}. For compressive testing, strain rates include 1-2Hz, 32%/s, and 0.5-100 mm/min\textsuperscript{64–67}. The tensile moduli have been reported as 100-300 MPa in the circumferential direction and 10 fold less in radial direction\textsuperscript{37,62}. The ultimate tensile strength has been reported as 5.3-16.8 MPa for circumferential and 1.18-2.95 MPa for radial direction\textsuperscript{62}. Both creep indentation and confined/unconfined compression tests can be used and have been reported an aggregate modulus of 0.10-0.40 MPa\textsuperscript{24,37}.

\textbf{Meniscus Replacement Options}

Meniscus replacement options are an active area of research. Typical strategies include top-down approaches, where native meniscus is processed for re-implantation, or bottom-up approaches, where a meniscus is created from scratch. Regardless of the strategy, meniscus replacements have two main problems: 1) viscoelasticity and complex fiber architectures are difficult to replicate in laboratory made materials, and 2) allografts are often not able to integrate with the joint to survive long-term\textsuperscript{68–70}.

Electrospinning is a bottom-up approach that is gaining attention, where a polymer solution is sent through a charged needle onto a grounded collecting device at varying speeds. This technique can create materials with varying fiber alignments and densities. Additionally, mixtures of polymers may be used in varying ratios to fine tune
mechanical properties. Both natural and synthetic fibers may be used, such as collagen, elastin, and poly-(lactide-co-glycolide).

Synthetic scaffolds include poly-glycolic acid (PGA) and poly-L-lactic acid (PLLA), where fibrochondrocytes seed well on PGA. While PGA scaffolds encourage vascularization and allow fibrocartilaginous scaffolds to form, their mechanical properties are typically not sufficient. Natural scaffolds include collagen meshes, hyaluronic acid, agarose, and decellularized tissue. More recently, scaffoldless approaches have been attempted where cells are seeded in an agarose mold which prevents attachment. The cells produce collagen I and II and proteoglycans, resulting in constructs that are 200-400% stiffer than PGA.

The Future of Meniscus Research

While the meniscus has been widely researched, a viable long-term replacement has yet to be available. Toward this goal, it is important to study the mechanics of the meniscus in various scenarios, including hydration fluid, tears, loss of cells, etc. It is also important to combine the efforts of previous meniscus scaffolds and constructs to lead to long-term replacement options. A meniscus replacement must first mimic the natural mechanical properties of the meniscus and withstand high loading. The replacement must also be able to survive in a potentially arthritic environment, with reduced synovial fluid viscosity and inflammation. The replacement must also integrate in this environment and incorporate native vasculature and nerves.

However, in order to test any potential solution to this complex problem, the effects of mechanical testing set-ups should be examined, and standardized mechanical testing should be implemented to maintain consistency across studies. Thus, the
following two chapters will investigate meniscus mechanics when hydrated in synovial fluid versus phosphate buffered saline and the effects of cell removal. The effects of increasing meniscus porosity will also be examined as a potential option for improving vascularization and innervation in allografts.
CHAPTER 3
COMPARING THE MECHANICAL PROPERTIES OF THE PORCINE KNEE MENISCUS WHEN HYDRATED IN SALINE VERSUS SYNOVIAL FLUID

Ex vivo Testing in Synovial Fluid

Like most tissues, the knee meniscus is viscoelastic and displays different behavior when dehydrated compared to hydrated\textsuperscript{83}. Thus, to fully understand meniscus mechanics, both solid and fluid constituents within the tissue should be considered. Within the knee, the menisci are hydrated by synovial fluid, which serves as a source of nutrition and lubrication\textsuperscript{84}. Yet, previous research on meniscus mechanics often uses phosphate-buffered saline (PBS) for tissue hydration\textsuperscript{24,63,67,85–88}. This substitution is typically motivated by the inhomogeneity of synovial fluid, as synovial fluid is physically a colloid consisting of multiple lubricating factors and large molecules dispersed throughout. However, PBS viscosity is only 0.001 Pa·s\textsuperscript{89}, whereas synovial fluid viscosity ranges from 0.08 to 1.9 Pa·s in healthy individuals\textsuperscript{90}. PBS is also a Newtonian fluid, while synovial fluid is a complex non-Newtonian fluid that demonstrates shear-thinning characteristics. Since PBS and synovial fluid are mechanically different fluids, the mechanics of meniscal tissue may differ when hydrated with these two fluids. The goal of this chapter is to assess the effect of these hydration fluids on meniscus mechanical properties.

\textsuperscript{1} This chapter previously published as: Lakes EH, Kline CL, McFetridge PS, Allen KD. Comparing the mechanical properties of the porcine knee meniscus when hydrated in saline versus synovial fluid. J Biomech 2015;48:4333-38. Used with permission.
Experimental Design: Testing in Synovial Fluid and PBS

Porcine medial menisci (n = 28) and bovine synovial fluid were purchased from Animal Technologies, Inc. (Tyler, Texas) and shipped frozen on dry ice. To test the effects of hydration fluid, a paired experiment was designed, where half the meniscus was tested in synovial fluid and the remaining half was tested in PBS (Figure 3-1). The assigned fluid was alternated to account for known regional variability within the meniscus\textsuperscript{24,87,88}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure3-1.png}
\caption{Meniscus sample preparation for mechanical testing in synovial fluid and PBS. Each meniscus was dissected in half to allow for one half to have samples tested in PBS and the other half to have samples tested in synovial fluid (SF). Fluid assignments were alternated between anterior/posterior regions among menisci. A) Tensile samples were taken as both radial and circumferential direction in relation to the circumferential collagen fibers. Dumbbell tensile samples had a central width of 2 mm and central length of 3.7 mm. B) For compression testing, two cylindrical samples were taken next to each other in the central portion of the meniscus and were then cut with parallel blades.}
\end{figure}
**Tension**

The halves of 12 different menisci were frozen to a cryostat stage and sliced to obtain a centrally-located, 450 µm thick section parallel to the tibial surface. Using a custom dumbbell-shaped punch, a sample aligned with the circumferential collagen fibers (circumferential) and a sample aligned perpendicular to the fibers (radial) were obtained from each half (Figure 3-1). Samples were stored in a 0.15 M NaCl solution with protease inhibitors (2 mM EDTA, 5 mM benzamidine HCl, 10 mM N-ethylmaleimide, and 1 mM PMSF)\(^{60,87,88}\). Prior to testing, samples were transferred to either synovial fluid or PBS for 48 h (at 4°C).

For tensile testing, samples were secured in hemostat grips on a 5542 model Instron (±50 N load cell) and immersed in PBS or synovial fluid at room temperature. Two lots of synovial fluid were used, with half of the samples tested in each lot. For circumferential samples, a 0.05 N tare load was applied; then, samples were preconditioned with 10 cycles of 0.65% strain followed by 10 testing cycles of 1.3% strain at 0.26%/s. For radial samples, a 0.05 N tare load was applied; then, samples were preconditioned with 10 cycles of 2.5% strain followed by 10 testing cycles of 5% strain at 1%/s. Strains and strain rates were selected from work reporting circumferential strains of 1.3% and radial strains of 5% in the knee meniscus after five seconds of a physiologic load\(^{59}\). Immediately after cycling, samples underwent pull to failure at the same strain rate. From cyclic loading, area of hysteresis and peak stress were calculated, and from pull to failure tests, Young's modulus, yield stress, yield strain, ultimate tensile strength (UTS), and strain at UTS were calculated. Young's modulus was defined as the linear portion of the stress–strain curve after the toe region.
Compression

Using a 5 mm biopsy punch, samples perpendicular to the tibial surface in the central portion of the meniscus were collected from the halves of 16 menisci (Figure 3-1). Samples were cut with parallel blades to a height of 3.5 mm, with both surfaces removed. As for tension testing, samples were placed in saline with protease inhibitors, then transferred to either synovial fluid or PBS for 48 h prior to testing (at 4°C).

For unconfined compression, samples were secured to a petri dish via cyanoacrylate, then surrounded by room temperature PBS or synovial fluid (5542 model Instron, ±500 N load cell). Three different lots of synovial fluid were used, with samples 1–3 tested in the first lot, sample 4 was tested in the second lot, and samples 5–16 tested in the third lot. Samples were cycled 30 times at 10% strain at a strain rate of 2.5%/s. The first 15 cycles were considered preconditioning and the last 15 cycles were considered the repeatable response for cyclic loading. Immediately after cycling, each sample underwent stress relaxation at 20% strain until a steady-state stress was reached (≈ 30 min). Cycling at 10% strain was based on an estimation of physiologic loading\(^9\). The strain rate was chosen from previous testing of meniscal attachments\(^8\) and preliminary testing showing higher strain rates associated with walking\(^6\) exceeded our machine’s capabilities. From cyclic loading, area of hysteresis and peak stress were calculated. Young’s modulus was calculated from the ramping phase between cycling and stress relaxation, while the instantaneous stress and relaxation stress were calculated from stress relaxation data.
**Stress-Relaxation Curve Fitting**

To further characterize stress relaxation responses, first-order decay and standard linear solid (SLS) models were fit to stress relaxation data. First-order decay provided an estimate of the time constant (63% decay from instantaneous stress). The SLS model is governed by the following equation:

\[
\sigma(t) = \varepsilon_0 \cdot (E_1 + E_2 e^{-t/\tau})
\]  

where \(E_1 + E_2\) is the instantaneous modulus, \(E_1\) is the relaxation modulus, and \(\tau\) is the time constant for relaxation\(^{92}\).

**Statistical Analysis Comparing Synovial Fluid and PBS**

Since PBS and synovial fluid samples had a matched sample from the same meniscus, a paired t-test was used to compare samples tested in PBS versus samples tested in synovial fluid (\(\alpha = 0.05\)). To compare the variance between groups, an F-test was performed (\(\alpha = 0.05\)). Due to an experimental error during test set-up, one radial and one circumferential tensile sample was excluded from analysis (dropping from \(n = 12\) to \(n = 11\)). Additionally, in compression, one meniscus sample was accidentally stored and tested in the incorrect fluid (dropping from \(n = 16\) to \(n = 15\) for compression). Lastly, failure and yield occurred at a similar stress–strain, thus while the yield stress and yield strain were calculated, only UTS and strain at UTS are presented.

**Results for Synovial Fluid and PBS Hydration in Tension**

For radial samples, no differences were found between PBS and synovial fluid in hysteresis area (\(p = 0.854\)) and peak stress during hysteresis cycling (\(p = 0.902\)) (Figure 3-2, next page). Additionally, hydration fluid did not affect the radial Young’s
modulus ($p = 0.362$), UTS ($p = 0.565$), or strain at UTS ($p = 0.995$) (Figure 3-3, next page). For circumferential samples, no differences were found in hysteresis area ($p = 0.507$) and peak stress during hysteresis cycling ($p = 0.760$) (Figure 3-2). Additionally, hydration fluid did not affect the circumferential Young’s modulus ($p = 0.362$), UTS ($p = 0.766$), or strain at UTS ($p = 0.862$) (Figure 3-3, next page). Finally, variability in tension parameters was not significantly affected by hydration fluid ($p \geq 0.077$).

Figure 3-2. Cyclic tensile results for meniscus tested in synovial fluid and PBS. The tensile hysteresis is shown for PBS and synovial fluid (SF) samples. A) hysteresis area for radial samples, B) peak stress for radial hysteresis cycles, C) graphical representation of hysteresis averages for radial samples, D) hysteresis area for circumferential samples, E) peak stress for circumferential hysteresis cycles, and F) graphical representation of hysteresis for circumferential samples.
Figure 3-3. Pull to failure results of tensile samples tested in PBS versus synovial fluid (SF), showing the A) Young’s modulus for radial samples, B) strain at ultimate tensile strength for radial samples, C) ultimate tensile strength for radial samples, D) Young’s modulus for circumferential samples, E) strain at ultimate tensile strength for circumferential samples, and F) ultimate tensile strength for circumferential samples.
Results for Synovial Fluid and PBS Hydration in Compression

Hydration fluid did not affect the mean value of hysteresis area ($p = 0.679$) or peak stress during cyclic compression ($p = 0.575$) (Figure 3-4). Similarly, no differences were found for the Young’s modulus ($p = 0.887$), instantaneous stress ($p = 0.778$), and relaxation stress ($p = 0.244$) (Figure 3-5, next page). However, the variability of all measured compressive properties was reduced in synovial fluid ($p \leq 0.022$). Graphical representation of average stress relaxation curves with standard deviation bounds is shown in Figure 3-6 (next page).

Figure 3-4. Hysteresis for compressive cyclic loading in PBS versus synovial fluid (SF), with the A) hysteresis area, B) peak stress for hysteresis cycles, and C) graphical representative of hysteresis averages. ♦ indicates significance found from F-test ($p < 0.05$).
Figure 3-5. Stress relaxation results of compression samples tested in PBS versus synovial fluid (SF), showing the A) Young’s modulus, B) instantaneous stress, and C) relaxation stress. ♦ indicates significance found from an F-test ($p < 0.05$).

Figure 3-6. Graphical representation of the average stress relaxation curves for samples tested in A) PBS and B) synovial fluid. Solid lines represent the average with ± 1 standard deviation error bounds.
Quantitatively, time constants from the first-order decay model were similar in different hydration fluids ($p = 0.245$), but lower variability was found in samples tested in synovial fluid ($p \leq 0.001$, Table 3-1). Similarly, for SLS curves, no differences were found for time constant ($p = 0.507$), instantaneous modulus ($p = 0.932$), or relaxation modulus ($p = 0.326$), but the variability of these measures was generally reduced in synovial fluid, with a significant reduction in the relaxation modulus ($p < 0.001$, Table 3-1).

Table 3-1. Stress relaxation values for PBS and synovial fluid (SF) samples are shown for the first-order decay and the standard linear solid curve fitting methods. ♦ indicates significance found from an F-test ($p < 0.05$)

<table>
<thead>
<tr>
<th></th>
<th>First-Order Decay</th>
<th>Standard Linear Solid Curve Fit</th>
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<tbody>
<tr>
<td></td>
<td>Tau</td>
<td>Tau</td>
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<tr>
<td>PBS</td>
<td>17.89 ± 12.47 ♦</td>
<td>44.14 ± 49.43</td>
</tr>
<tr>
<td>SF</td>
<td>14.04 ± 3.58 ♦</td>
<td>33.41 ± 33.91 ♦</td>
</tr>
</tbody>
</table>

Discussion of Synovial Fluid versus PBS Hydration

Menisci did not exhibit different mechanical behavior when hydrated in PBS versus synovial fluid; however in compression, variability was lower when testing samples in synovial fluid. These differences are likely driven by fluid–solid interactions during compression. In tension, collagen fibers take most of the load; thus, fluid within the tissue is likely not as influential. However, in compression, initial incompressibility of the fluid and drag of the fluid against the solid matrix plays a larger role.

Given the consistency of PBS and relative inhomogeneity of synovial fluid, higher variability was actually anticipated for samples tested in synovial fluid. Synovial fluid
viscosity can vary across a large range and is influenced by age, temperature, agitation, and rate of dispersion during preparation\textsuperscript{93}. Our bovine synovial fluid source combines synovial fluid from several animals, and pooling samples from multiple animals likely lowers variability between synovial fluid lots. Thus, even though multiple lots were used, the differences between synovial fluid lots are unlikely to represent inter-individual variability in synovial fluid mechanics. As such, the experiment did not examine the effects of changes in synovial fluid mechanics on meniscus mechanics.

Regardless, our results show reduced variability during compression testing in synovial fluid compared to PBS. This may be explained by how lubricating properties of synovial fluid allow fluid to flow more consistently through the tissue, by differences in how PBS and synovial fluid penetrate the tissue, or by other unknown causes. Sample dimensions acquired immediately prior to testing were similar for PBS and synovial fluid groups (data not shown), indicating swelling was not appreciably different between PBS and synovial fluid over 48 h.

In stress relaxation, fluid exudes from the tissue and stress lowers to a steady-state response. Since fluid has ceased moving at steady state, load should be carried by the solid matrix. Thus, one would expect hydration fluid to have minimal effect on material properties at steady-state; however, in this study, the variability of the relaxation modulus was decreased for synovial fluid. One potential explanation is synovial fluid allows collagen fibers to slide more easily along each other, leading to a more consistently relaxed tissue (lower relaxation stress in Figure 3-5). In PBS, internal friction between fibers may be present, leading to higher relaxation moduli and increased variability.
Since the meniscus is a complex viscoelastic tissue, literature values on meniscus mechanics vary based on species, age, and testing protocols. Our data align well with prior reports on the porcine meniscus, where circumferential UTS was 29.60 MPa when pulled at 1.6%/s\textsuperscript{94}. However, strain at UTS in this prior report was 64.45%, while our strain at UTS was 36.3 ± 6.2%. For compression, prior stress relaxation work in the porcine meniscus reports an instantaneous stress of 5.0 MPa when loaded to 50% strain\textsuperscript{95}. Instantaneous stress in this study was lower (1.18 ± 0.89 MPa), but our constructs were loaded to 20% strain. Given the strain stiffening potential of meniscal tissues\textsuperscript{96}, these differences are not unlikely for knee meniscus samples at different strains. Expanding to humans, menisci are reported to have a circumferential modulus of 90.22 MPa and radial modulus of 11.49 MPa\textsuperscript{97}. Again, our tensile results align well with prior reports for the pig and demonstrate reasonably similar meniscus properties between the human and pig.

Importantly, the results of this study indicate the mean tensile and compressive properties of the meniscus are largely independent of testing fluid, indicating past experiments conducted in PBS likely yielded an accurate approximation of the mean mechanical properties. However, more consistent compression results may be obtained if samples are tested in synovial fluid. While the reasons for the decrease in variability are currently unknown, future work may provide more insight, and the use of synovial fluid in compression testing may decrease the necessary sample size or increase the power of statistical analysis in future experiments.
CHAPTER 4
MECHANICAL INTEGRITY OF A DECELLULARIZED AND LASER DRILLED MEDIAL MENISCUS

Meniscus Replacements

Resection of the knee meniscus is one of the most common orthopedic surgeries in the U.S., with approximately 1 million procedures performed each year. These procedures typically provide pain relief for patients; however, meniscus resection is known to increase the risk of OA development by four-fold. A clinically relevant long-lasting meniscus replacement following meniscectomy may help to reduce or prevent the progression of OA in patients with damaged menisci. To date, research on meniscal replacements has focused on methods to recreate the complex structure and mechanical function of the knee meniscus, including efforts to generate allogenic, xenogeneic, and synthetic replacements, as well as tissue engineering approaches to regenerate a living and biologically functional meniscus replacement.

Tissue engineering approaches include the use of synthetic scaffolds, the scaffoldless generation of meniscal replacements in negative molds, and the use of aligned electrospun scaffolds. Cell-seeded, nonwoven PGA (polyglycolic acid) scaffolds have been shown to encourage vascularization and fibrocartilaginous tissue growth, but mechanical properties are not yet sufficient for physiological loading. Scaffoldless approaches, where cells are seeded in a negative agarose mold to encourage cell-to-cell interactions, are able to produce neotissue constructs that are composed entirely of natural proteins and are 200–400% stiffer than constructs.

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formed on PGA\textsuperscript{37,81,82}. However, these constructs require extensive development through cell culture, which is both labor intensive and expensive. Finally, electrospinning synthetic materials, such as polycaprolactone and poly-L-lactic acid (PLLA), allows for control of material properties and basic fiber alignment\textsuperscript{103}, but this technique has yet to produce a fully formed construct that mimics the complex fiber alignment of the knee meniscus.

Natural scaffolds and materials are also being developed for meniscal replacements, including collagen meshes, hyaluronic acid, and decellularized tissue; but, these tissues require further characterization for meniscal tissue engineering purposes\textsuperscript{18}. Meniscus allograft approaches commonly use cryopreserved, fresh-frozen, or occasionally lyophilized tissues from cadaveric donors, with failure rates for these meniscal allografts between 10% and 29%\textsuperscript{104}. Studies of cryopreserved and fresh-frozen meniscus allografts show survival between 2 and 7 years; however, in one study, 21.3% of successes still required a second surgery to repair tears in the allograft\textsuperscript{68,70}. While fresh-frozen and cryopreserved tissues are typically not decellularized via a detergent, fresh-frozen grafts may undergo gamma irradiation, and cryopreserved grafts only leave 10% of cells viable\textsuperscript{69,105}. Even so, fresh-frozen allografts may cause immune responses\textsuperscript{106,107}; thus, an acellular graft may be beneficial. Of the options available for natural meniscal replacements, decellularized allografts are a cost effective and clinically relevant option that maintains the complex fiber architectures needed for physiologic loading. However, allografts are currently unable to withstand long-term physiologic loading with failure rates increasing to 45% at 10 years\textsuperscript{68,69}. Hence, a middle ground that borrows the initial mechanical integrity of allografts and the long-term
remodeling potential of tissue engineered constructs may improve our ability to provide a long term replacement for an injured meniscus.

Prior work has shown increasing the porosity of scaffolds improves fibrocartilaginous ingrowth and integration with native tissue; however, the materials in these studies have primarily been synthetic rather than native tissue. In a study using tissue from the temporomandibular joint (TMJ) disk, increased cellular integration was achieved by creating a laser micropattern to improve transport into the decellularized tissue. Since the knee meniscus has similar characteristics to the TMJ disk, increasing the porosity of decellularized meniscal allografts without a significant reduction in mechanical properties could provide initial advantages under physiologic loading. This strategy showed promising results in a chemically etched ovine meniscus where small openings were created in a decellularized tissue. However, this study created pores ranging from 20 to 50 µm wide, while previously mentioned studies in synthetic materials and the TMJ disk found that macropores ranging from 120 to 500 µm are ideal for fibrocartilaginous ingrowth and cellular infiltration. Thus, a native meniscus allograft with pores ranging from 120 to 500 µm may represent a “sweet spot,” where sufficient mechanical integrity is retained for short term survival while fibrocartilaginous and vascular ingrowth is encouraged for long term health. Additionally, in order to have the greatest potential for widespread clinical use, an acellular porous meniscus scaffold would be most cost effective and least time consuming; however, a porous meniscus replacement could also be reseeded prior to implantation.
As a first step toward this long-term goal, this chapter investigates the mechanical integrity of a decellularized meniscus both before and after laser drilling. While it is expected mechanical properties will change when introducing pores into the tissue, this chapter aims to experimentally test how much alteration of the mechanical properties occurs and whether these changes are physiologically relevant. Future studies will determine the benefit of this procedure and if implanting this tissue as an acellular or recellularized construct is best.

Materials and Methods for Medial Meniscus Testing

Medial Meniscus Sample Preparation

Medial porcine menisci (n = 48) were obtained from Animal Technologies (Tyler, TX). The supplier stated that the exact age of the animal could not be confirmed, but samples were typically obtained from 6 to 9 month old pigs. Menisci arrived frozen at 20°C, and upon arrival, samples were wrapped in gauze soaked with a 0.15 M NaCl solution containing protease inhibitors (2 mM EDTA, 5 mM benzamidine HCl, 10 mM N-ethylmaleimide, and 1 mM PMSF) and stored at 20°C prior to use. Menisci were used to separately evaluate the effects of decellularization and the effects of laser drilling, whereby half of the menisci were used to test decellularized tissue relative to native tissue and half were used to test laser drilled tissue relative to decellularized tissue.

Decellularization Process

To evaluate the effects of decellularization, 24 menisci were dissected into anterior and posterior halves, where one half was used to represent native tissue while the other half was decellularized (Figure 4-1, next page). For decellularization,
meniscus samples were placed in 0.1% SDS (diluted in deionized water) at room temperature for 48 hrs (with two changes) followed by 10 rinses in deionized water and four rinses in phosphate buffered saline (PBS). SDS was chosen based on prior work in the TMJ disk which showed SDS maintains mechanical properties of the tissue better than Triton X-100 or ethanol/acetone\textsuperscript{116}. Decellularization was confirmed in a separate set of menisci that underwent hematoxylin and eosin and 4',6-diamidino-2-phenylindole (DAPI) staining (Figure 4-2, next page); 10 µm sections were obtained on a cryotome and stained using standard histological procedures. Tensile testing was performed on 12 menisci, and compression testing was performed on the other 12 menisci.

Figure 4-1. Meniscus preparation for mechanical testing. All menisci were dissected in half to allow for different treatments within the same meniscus (native/decellularized or decellularized/laser drilled). A) Tensile samples were taken as both radial and circumferential in relation to the circumferential collagen fibers of the meniscus. B) For compression testing, two cylindrical samples were taken next to each other in the central portion of the meniscus. Samples were then cut with parallel blades.
Figure 4-2. Histological staining of native, decellularized, and laser drilled meniscus. Top: Hematoxylin and eosin staining for A) native, B) decellularized, and C) laser drilled menisci. Bottom: DAPI staining for D) native, E) decellularized, and F) laser drilled menisci. Slices for all staining were taken at 10 µm on a cryotome. G) Macro view of a laser drilled meniscus showing full thickness pores. Photo courtesy of Andrea Matuska.

**Laser Drilling of Meniscus**

To evaluate the effects of laser drilling, an additional 24 menisci were dissected into anterior and posterior halves and decellularized via SDS, as described previously. One half of the meniscus remained decellularized, while the other half was processed for laser drilling. For laser drilling, menisci halves were either sliced at 450 µm or cut into a cylindrical sample and lyophilized in a benchtop manifold freeze dryer at 80°C.
(Millrock Technologies, Kingston, NY). Then, laser drilling was accomplished with a CO\textsubscript{2} laser engraver, creating a 1.4 mm spaced grid pattern at the lowest power setting (power = 7.2\%, irradiation time = 0.3–2.5 s, Hurricane Lasers, Las Vegas, NV). Preliminary trials confirmed the laser setting would produce full thickness holes through each sample (Figure 4-2G). Microscopic measurements confirmed an average diameter of 263.53 ± 27.21 \(\mu\)m and spacing of 1.27 ± 0.051 mm in samples used for tensile testing and an average diameter of 234.94 ± 75.03 \(\mu\)m and spacing of 1.39 ± 0.083 mm in samples used for compressive testing. Diameter of holes was chosen based on prior work showing macropores ranging from 120 to 500 \(\mu\)m in synthetic meniscus scaffolds and naturally derived temporomandibular disk scaffolds\textsuperscript{108,109,111,113}. Similarly, spacing of holes was chosen based on prior work showing cellular infiltration 1 mm deep into a meniscal scaffold\textsuperscript{117–119}, thus, it was assumed that a diagonal spacing of 2 mm should allow cells to adequately infiltrate the tissue in future studies. A separate set of laser drilled menisci also underwent histological staining with hematoxylin and eosin or DAPI to confirm decellularization (Figure 4-2, shown previously). Tensile testing was then performed on 12 menisci and compression testing was performed on the other 12 menisci.

**Tension Sample Preparation and Testing Procedure**

For tensile testing, meniscal halves were frozen to the stage of a cryostat at 20°C and sliced parallel to the tibial surface at a thickness of 450 \(\mu\)m. In an attempt to reduce sample variation due to differences in depth, a slice was acquired from the central depth of each meniscus (Figure 4-1a). Slices were then cut into tensile samples using a custom dumb bell shaped punch (central width = 2 mm and central length = 3.7 mm).
One sample was aligned with the circumferential axis of the collagen fibers (circumferential), while a second sample was aligned radially across the fiber direction (radial) (Figure 4-1A). Radial samples were placed near the dissection edge such that native/decellularized and decellularized/laser drilled samples were both from the central region of the same meniscus. Circumferential samples were placed in the anterior or posterior regions of the meniscus, and to account for known regional variability\textsuperscript{24,87}, the treatment of anterior and posterior meniscal halves (native/decellularized or decellularized/laser drilled) was alternated in different menisci. Prior to testing, tension samples were stored in NaCl solution with protease inhibitors at 4°C for 1–11 days.

For tensile testing, samples were first placed in hemostat grips on a 5542 model Instron and immersed in a room temperature PBS bath. After the application of a 0.05 N tare load, circumferential samples were preconditioned with 10 cycles of 0.65\% strain followed by 10 cycles of 1.3\% strain at a rate of 0.26\%/s. Similarly, after a 0.05 N tare load, radial samples were preconditioned with 10 cycles of 2.5\% strain followed by 10 cycles of 5\% stain at 1\%/s. All strain values and rates were based on prior work reporting tensile circumferential strains of 1.3\% and radial strains of 5\% over a period of 5 s in the meniscus\textsuperscript{59}. Immediately after cycling in both circumferential and radial samples, pull to failure testing was performed at the same strain rate. In these tests, clamp-to-clamp strain was measured and the cross-sectional area was assumed to be 450 µm (section thickness) by 2.2 mm (width of the central portion of the punch). From the collected data, the area of hysteresis for cyclic loading, peak stress for hysteresis cycles, loading energy, Young’s modulus, yield stress, yield strain, ultimate tensile
strength (UTS), and strain at UTS were evaluated. Note that the Young’s Modulus was measured as the linear portion of the stress–strain curve during pull to failure.

**Compression Sample Preparation and Testing Procedure**

For compression testing, meniscal halves were cut perpendicular to the tibial surface using a 5 mm diameter biopsy punch (Figure 4-1B). As with radial tensile testing, samples were taken near the dissection edge such that samples in both treatments (native/decellularized and decellularized/laser drilled) were from the central region of the same meniscus. Anterior/posterior assignments were still alternated between menisci as an additional precaution against regional variability. Samples were then cut with parallel microtome blades separated by 3.5 mm such that both the superior and inferior surfaces of the meniscus were removed and a consistent aspect ratio was maintained between samples. Prior to testing, compression samples were stored in NaCl solution with protease inhibitors at 4°C prior to testing for 1–3 days.

For compression testing, cylindrical samples were secured to a petri dish using cyanoacrylate, then surrounded by room temperature PBS. After the application of a 0.05 N tare load, samples were loaded to 10% strain at 2.5%/s for 30 cycles followed by a stress–relaxation test at 20% strain. Cycling at 10% strain was an estimation of physiologic loading of the meniscus and has been used in previous compression bioreactors. The strain rate was chosen based on previous mechanical testing of meniscal attachments, as well as preliminary tests demonstrating cyclic strain rates representative of walking exceeded the capabilities of our testing machine. Each sample was cycled 30 times, where the initial 15 cycles were taken as preconditioning and the last 15 cycles were the steady-state cyclic response. In these tests, clamp-to-
clamp strain was measured and the diameter was measured with digital calipers prior to testing. From these tests, the area of hysteresis for cyclic loading, peak stress for hysteresis cycles, loading energy, Young’s modulus, instantaneous stress at the initiation of stress relaxation, and the steady-state stress after stress relaxation were calculated. Note that the Young’s Modulus was measured as the linear portion of the stress–strain curve when ramping to 20% strain for stress relaxation.

**Stress–Relaxation Curve Fits**

To further characterize stress relaxation, a first-order decay model was used to estimate the time constant (time to 63% decay from instantaneous stress). Then, as a second estimation, stress relaxation curves were fit to the standard linear solid (SLS) model governed by the following equation:

\[
\sigma(t) = \varepsilon_0 \cdot (E_1 + E_2 e^{-t/\tau})
\]

(4-1)

where \(\varepsilon_0\) is the initial strain at the start of stress–relaxation, \(E_1 + E_2\) is the instantaneous modulus, \(E_1\) is the steady-state modulus, and \(\tau\) is the time constant.

Finally, as a third characterization of the stress relaxation, a quasi-linear viscoelastic (QLV) model was fit to each stress relaxation curve. Following QLV theory proposed by Fung, when a step strain of \(K\) is applied to a tissue, the relaxation function can be assumed to be:

\[
K[\lambda; t] = G(t) \times T^e(\lambda)
\]

(4-2)

where \(G(t)\) is the reduced relaxation function and \(T^e(\lambda)\) is the elastic response\(^{121}\). \(G(t)\) was defined as a series of three exponentials\(^{122-124}\)

\[
G(t) = ae^{-bt} + ce^{-dt} + ge^{-ht}
\]

(4-3)
where $a$, $c$, and $g$ are coefficients and $b$, $d$, and $h$ are exponential terms. Finally, $T^\theta(\lambda)$ was defined as$^{122,123}$

$$T^\theta(\lambda) = A(e^{B\lambda} - 1)$$

QLV equations were fit using a least squares algorithm (MATLAB, Version 7.13, The Mathworks, Natick, MA) where starting points for $A$, $B$, $a$, $b$, $c$, $d$, $g$, and $h$ were randomly generated numbers between 0 and 1 and bounds were set such that all coefficients were greater than 0, parameters $A$, $B$, $a$, $c$, and $g$ were constrained to an upper limit of 10, and the exponential terms $b$, $d$, and $h$ were more tightly constrained to an upper limit of $2^{122}$. For each sample, the QLV model was fit to the stress relaxation data five times, with randomly chosen starting points each time. Then, an average value for each coefficient was calculated from the five separate QLV fits, with the coefficient of variation (CV) across fits calculated to represent the repeatability of the QLV parameter estimates. Inter-animal variability was calculated by investigating the standard deviation of the average values obtained for different meniscal samples.

**Statistical Analysis Comparing Native, Decellularized, and Laser Drilled Meniscus**

To assess the decellularization process (native/decellularized) and the laser drilling process (decellularized/laser drilled), experiments were designed such that paired samples existed for each treatment. Thus, a paired t-test was conducted to investigate differences between native and decellularized and between decellularized and laser drilled tissues ($\alpha = 0.05$). Since the goal of the experiment is to demonstrate minimal loss of mechanical integrity, Bonferroni corrections were not applied for any comparison, as accepting a higher type I error rate is the conservative position when the scientific objective aligns with the null hypothesis (no loss of mechanical integrity).
For tensile testing of native versus decellularized samples, the hysteresis calculations of both radial and circumferential samples in half of the menisci were excluded due to issues with load cell sensitivity (n = 12 becomes n = 6 for hysteresis calculations), and two radial samples were lost during preparation for native/decellularized calculations (n = 5 for hysteresis and n = 10 for pull to failure). Additionally, for tensile testing, the yield stress and yield strain were calculated, but since values occurred close to failure, only UTS and strain and UTS were statistically analyzed. Finally, for all laser drilled samples, the loss of tissue was accounted for in all stress calculations.

Native versus Decellularized Results

Radial Tension Results: Native versus Decellularized

For radial tension samples, the area of hysteresis and loading energy tended to decrease, but showed no significant differences for native versus decellularized tissues (p = 0.093 and p = 0.113 respectively, Figures 4-3A and 4-3B, next page). However, the peak stress of hysteresis was significantly decreased from a native mean of 0.39 ± 0.13 MPa to a decellularized mean of 0.22 ± 0.07 MPa (p = 0.049, Figure 4-3C). This change can also be visualized in Figure 4-3D where a decrease in the peak of the hysteresis curve is seen. Pull to failure tensile testing showed no significant differences in the Young’s modulus, strain at UTS, and UTS as visualized in Figures 4-3E-G where lines connecting samples from the same menisci alternate between positive and negative slopes, indicating the effects of decellularization are marginal relative to the variability between tests.
Figure 4-3. Native and decellularized tissue results from radial tensile testing showing the following: A) hysteresis area from cyclic loading, B) loading energy from cyclic loading, C) peak stress from cyclic loading, D) representative curves from cyclic loading, E) Young’s modulus from the linear portion of the pull to failure curve, F) strain at UTS, and G) UTS. Lines connect samples from the same meniscus, and the legend indicates whether samples from the anterior or posterior region of the meniscus. * = significance from paired t-test (p < 0.05).

Circumferential Tension Results: Native versus Decellularized

For circumferential samples, the tensile hysteresis area was not significantly different after decellularization ($p = 0.179$, Figure 4-4A). Loading energy and peak stress were unchanged with decellularization ($p = 0.236$ and $p = 0.553$, respectively,
Figures 4-4B-C). Additionally, no differences were seen in the pull to failure testing of circumferential sample, as visualized by the alternating lines connecting samples in Figures 4-4E-G.

Figure 4-4. Native and decellularized tissue results from circumferential tensile testing showing the following: A) hysteresis area from cyclic loading, B) loading energy from cyclic loading, C) peak stress from cyclic loading, D) representative curves from cyclic loading, E) Young’s modulus from the linear portion of the pull to failure curve, F) strain at UTS, and G) UTS. Lines connect samples from the same meniscus, and the legend indicates whether samples from the anterior or posterior region of the meniscus.
Compression Results: Native versus Decellularized

Cyclic compression testing showed a decrease in the hysteresis area, loading energy, and peak stress of hysteresis after decellularization ($p \leq 0.020$), as visualized by the negative slopes in Figures 4-5A-C and the lowered hysteresis curve in Figure 4-5D (next page). Additionally, the Young’s modulus during compressive loading, the instantaneous stress at the initiation of stress relaxation and the steady-state stress at the end of relaxation were also decreased after decellularization ($p \leq 0.023$, Figures 4-5E-G). The additional characteristics of stress relaxation are shown in Table 4-1 with the first-order decay estimation of the time constant and the SLS and QLV curve fitting results. The first-order decay derived time constant was decreased after decellularization ($p = 0.002$); however, the SLS derived time constant was not significantly decreased ($p = 0.829$). The SLS derived instantaneous modulus was unchanged, but the steady-state modulus was significantly decreased after decellularization ($p = 0.007$).
Figure 4-5. Native and decellularized tissue results from compressive testing showing the following: A) hysteresis area from cyclic loading, B) loading energy from cyclic loading, C) peak stress from cyclic loading, D) representative curves from cyclic loading, E) Young’s modulus from the linear portion of the loading phase, F) instantaneous stress at the beginning of stress relaxation, and G) steady-state stress after stress relaxation. Lines connect samples from the same meniscus, and the legend indicates whether samples from the anterior or posterior region of the meniscus. * = significance from paired t-test (p < 0.05).
Table 4-1. Native and decellularized stress relaxation values showing the average 6 inter-animal standard deviation for the first-order decay estimation of the time constant, the SLS, and QLV curve fits. In addition, a CV for each QLV coefficient was calculated from the five different fits performed on each sample. Then, the average CV for each coefficient was calculated to represent the repeatability of the QLV fit. * = significance from paired t-test (p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>First-Order Decay</th>
<th>Standard Linear Solid Curve Fit</th>
<th>Quasi-Linear Viscoelastic Curve Fit</th>
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<tr>
<td></td>
<td>Tau</td>
<td>E&lt;sub&gt;Instantaneous&lt;/sub&gt;</td>
<td>E&lt;sub&gt;Relaxation&lt;/sub&gt;</td>
</tr>
<tr>
<td>Native</td>
<td>7.27 ± 1.65</td>
<td>8.61 ± 4.48</td>
<td>3.77 ± 1.77</td>
</tr>
<tr>
<td>Decellularized</td>
<td>5.25 ± 0.89</td>
<td>7.79 ± 10.35</td>
<td>2.02 ± 2.12</td>
</tr>
</tbody>
</table>

Finally, for QLV curve fitting, significant changes were seen between native and decellularized tissues in parameters A (p = 0.021), B (p = 0.01), b (p = 0.027), d (p = 0.007), and g (p = 0.042). The repeatability of the QLV fits is represented by the average CV for each QLV parameter. For elastic parameters A and B, QLV fits were reasonably consistent, independent of the start point. However, for relaxation function parameters a, b, c, d, g, and h, the CV was greater than 100%, indicating the estimation of the parameter was relatively inconsistent across fits.
Decellularized versus Laser Drilled Results

Radial Tension Results: Decellularized versus Laser Drilled

For radial tension samples, the area of hysteresis, loading energy, and peak stress of hysteresis showed no significant differences for decellularized versus laser drilled tissues (p ≥ 0.127, Figures 4-6A-D). Additionally, pull to failure tensile testing showed no significant differences in the Young’s modulus, strain at UTS, and UTS (p ≥ 0.116, Figures 4-6E-G, next page).

Circumferential Tension Results: Decellularized versus Laser Drilled

For circumferential samples, the tensile hysteresis area, loading energy, and peak stress were not significantly different (p ≥ 0.365, Figures 4-7a–4-7d, page 56). There was a trend toward increased hysteresis area after laser drilling, which seems to be associated with increased stress dissipation at lower strains. This may be associated with the ability of the extracellular matrix to deform into the voids created by the laser drilling at low strains. For pull to failure testing, no differences were found for the Young’s modulus and strain at UTS, but a significant decrease in the UTS was found between a decellularized mean of 26.64 ± 6.10 and an laser drilled mean of 18.18 ± 7.54 (Figure 4-7G), as visualized by largely negative slopes connecting these groups (p = 0.036).
Figure 4-6. Decellularized and laser drilled tissue results from radial tensile testing showing the following: A) hysteresis area from cyclic loading, B) loading energy from cyclic loading, C) peak stress from cyclic loading, D) representative curves from cyclic loading, E) Young’s modulus from the linear portion of the pull to failure curve, F) strain at UTS, and G) UTS. Lines connect samples from the same meniscus, and the legend indicates whether samples from the anterior or posterior region of the meniscus.
Figure 4-7. Decellularized and laser drilled tissue results from circumferential tensile testing showing the following: A) hysteresis area from cyclic loading, B) loading energy from cyclic loading, C) peak stress from cyclic loading, D) representative curves from cyclic loading, E) Young’s modulus from the linear portion of the pull to failure curve, F) strain at UTS, and G) UTS. Lines connect samples from the same meniscus, and the legend indicates whether samples from the anterior or posterior region of the meniscus. * = significance from paired t-test (p < 0.05).
Compression Results: Decellularized versus Laser Drilled

Laser drilling did not affect the cyclic hysteresis properties relative to decellularized tissue \((p \geq 0.172, \text{Figures 4-8A-D, next page})\). Laser drilling reduced the Young's modulus and instantaneous stress relative to decellularized tissue \((p \leq 0.029, \text{Figures 4-8E and 4-8F})\), but did not reduce the steady-state modulus. The first-order decay estimation of the stress relaxation time constant is summarized in Table 4-2 (page 59), along with results from SLS and QLV curve fits. The first-order decay and SLS methods did not show differences between decellularized and laser drilled time constants for stress relaxation. However, the SLS derived instantaneous modulus revealed differences between decellularized and laser drilled \((p = 0.002)\) samples. Additionally, the QLV model showed differences in parameters \(A (p = 0.025), B (p = 0.008), \text{and} h (p = 0.032)\). Again, the repeatability of the QLV fits demonstrated reasonable consistency in the elastic response \((A, B)\); however, estimation of the relaxation function was relatively inconsistent across fits (CV for parameters \(a, b, c, d, g, \text{and} h\) greater than 100%).
Figure 4-8. Decellularized and laser drilled tissue results from compressive testing showing the following: A) hysteresis area from cyclic loading, B) loading energy from cyclic loading, C) peak stress from cyclic loading, D) representative curves from cyclic loading, E) Young’s modulus from the linear portion of the loading phase, F) instantaneous stress at the beginning of stress relaxation, and G) steady-state stress after stress relaxation. Lines connect samples from the same meniscus, and the legend indicates whether samples from the anterior or posterior region of the meniscus. * = significance from paired t-test (p < 0.05).
Table 4-2. Decellularized and laser drilled stress relaxation results showing the average 6 inter-animal standard deviation results for the first-order decay estimation of the time constant, the SLS, and QLV curve fits. In addition, a CV for each QLV coefficient was calculated from the five different fits performed on each sample. Then, the average CV for each coefficient was calculated to represent the repeatability of the QLV fit. * = significance from paired t-test (p < 0.05).

<table>
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<tbody>
<tr>
<td></td>
<td>Tau</td>
<td>Tau</td>
<td>E_{\text{Instantaneous}}</td>
</tr>
<tr>
<td>Decellularized</td>
<td>9.52 ± 10.86</td>
<td>30.85 ± 70.42</td>
<td>4.95 ± 3.37</td>
</tr>
<tr>
<td>Laser Drilled</td>
<td>10.11 ± 3.79</td>
<td>33.51 ± 33.23</td>
<td>0.89 ± 0.91</td>
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<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>a</th>
<th>b</th>
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<tr>
<td>Decellularized</td>
<td>0.21 ± 0.07</td>
<td>0.40 ± 0.11</td>
<td>2.45 ± 1.52</td>
<td>0.23 ± 0.19</td>
</tr>
<tr>
<td>(CV = 55%)</td>
<td></td>
<td>(CV = 22%)</td>
<td>(CV = 147%)</td>
<td>(CV = 128%)</td>
</tr>
<tr>
<td>Laser Drilled</td>
<td>0.15 ± 0.04</td>
<td>0.26 ± 0.09</td>
<td>2.34 ± 1.53</td>
<td>0.10 ± 0.08</td>
</tr>
<tr>
<td>(CV = 59%)</td>
<td></td>
<td>(CV = 29%)</td>
<td>(CV = 138%)</td>
<td>(CV = 148%)</td>
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<th>c</th>
<th>d</th>
<th>g</th>
<th>h</th>
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<tbody>
<tr>
<td>Decellularized</td>
<td>2.45 ± 2.23</td>
<td>0.20 ± 0.21</td>
<td>3.74 ± 2.08</td>
<td>0.27 ± 0.17</td>
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<tr>
<td>(CV = 145%)</td>
<td></td>
<td>(CV = 135%)</td>
<td>(CV = 137%)</td>
<td>(CV = 123%)</td>
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<tr>
<td>Laser Drilled</td>
<td>3.84 ± 2.91</td>
<td>0.14 ± 0.12</td>
<td>2.98 ± 2.63</td>
<td>0.12 ± 0.13</td>
</tr>
<tr>
<td>(CV = 111%)</td>
<td></td>
<td>(CV = 109%)</td>
<td>(CV = 115%)</td>
<td>(CV = 123%)</td>
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**Discussion of Native, Decellularized, and Laser Drilled Meniscus**

While the long-term goal of this project is to combine natural scaffolds and tissue engineering practices to generate a viable meniscal replacement, the goal of this study was to evaluate the feasibility of retaining the mechanical integrity in meniscal tissue during a decellularization and laser-drilling process as a first step toward creating an acellular porous meniscus scaffold. Of course, some loss of mechanical integrity is anticipated with the removal of cells via a detergent and with the physical disruption of
the extracellular matrix via laser drilling. Thus, observed changes in both tensile and compressive properties are not surprising. However, it is also worth noting that for all material properties tested, the decellularized and laser drilled constructs generally fell within the range of their comparison tissue. Thus, although some statistical significance was found, these changes may not be clinically meaningful, as discussed in the following paragraphs. Moreover, with optimization, the laser drilling pattern may be less disruptive to the material properties of the meniscus. Nonetheless, these data indicate there may be a sweet spot where the porosity of decellularized allografts can be increased without a significant reduction in mechanical properties, thereby retaining sufficient mechanical integrity in a meniscal scaffold while encouraging fibrocartilaginous and vascular ingrowth for long-term health.

Even though some mechanical parameters were statistically significant between groups, the physiological significance of these differences must also be considered. First, even in parameters where significant changes were detected (such as the compressive hysteresis parameters shown in Figure 4-5), the altered values still fell within the range of the comparison group. Thus, the changes seen in this study were not severe enough to cause great discrepancies and shifts between groups, and it may be possible to prescreen tissues for their suitability to serve as laser drilled meniscal scaffolds using nondestructive mechanical testing. Second, while changes in mechanical properties may be initially concerning, it is important to consider the physiological relevance of these findings. For example, in this study, the average instantaneous modulus decreased from a decellularized value of $4.95 \pm 3.38$ MPa to a laser drilled value of $0.89 \pm 0.91$ MPa (82% decrease). However, in a finite element
model of the knee meniscus, Meakin et al. reported compressive axial stress of 0.24 MPa under physiologic load\textsuperscript{57}, which is comparable to the instantaneous stress observed in laser drilled samples compressed to 20% strain (0.251 ± 0.24 MPa). Of course, species differences must also be considered, and while some laser drilled menisci would clearly struggle to provide meaningful support with an 82% decrease in compressive resistance, some menisci may still provide a physiologically relevant function. Furthermore, Meakin et al. also showed that axial displacements and stresses are more sensitive to tissue geometry than material properties, in which case allografts may have an advantage over other tissue engineering methods due to existing physiologic geometries\textsuperscript{57}.

Similarly, laser drilling of decellularized meniscus tissue also did not significantly alter the tensile pull to failure mechanics of radial samples and only lowered the UTS of circumferential samples. Again, while statistically significant, the decrease in UTS after laser drilling may not indicate a significant increased risk of failure under basic physiologic conditions. Circumferential stress in the meniscus has been estimated at 1.8–2.7 MPa\textsuperscript{59}, and laser drilled tissue did not fail until an average of 18.2 ± 7.54 MPa. Thus, while the factor of safety is reduced, this change does not imply a guaranteed failure, and some of this loss may be recovered through cell seeding and infiltration processes.

For fibrocartilaginous ingrowth, Groot et al. reported ideal macropore sizes of 200–300 µm for porous polyurethane (PU) and PLLA meniscal implants\textsuperscript{111}. Similar studies agree that synthetic meniscal implants with pore sizes between 150 and 500 µm being ideal for ingrowth\textsuperscript{108,112}, with 50–200 µm diameter holes being best when the
strength of fibrous tissue is important\textsuperscript{108,109}. Additionally, Juran et al. showed that laser drilling 120 µm diameter holes into a decellularized temporomandibular disk resulted in a construct with similar compressive properties to native tissue\textsuperscript{113}. In this study, laser drilling pore diameters ranged from 234.94 ± 75.03 µm for compression samples and 263.53 ± 27.21 µm for tensile samples, which agrees with previous findings in synthetic materials. However, the larger diameter holes used in this study may explain why this study found significant compressive changes, while studies such as Juran et al. did not.

For the future of laser drilled natural meniscus scaffolds, additional research may be needed to optimize laser drilling patterns such that increased porosity of the tissue is achieved while maintaining strength in the best possible way. Optimization of the pore size could be further advanced by studying the local stress/strain fields in the tissue and around the laser drilled holes. Moreover, the laser creates a conical beam, and as such, the hole diameters (and stress concentrations) can differ as a function of depth from the surface. Thus, optimization as a function of hole depth may also need to be considered. Nevertheless, this work serves as a proof of feasibility for this approach and further optimization of the hole pattern will be the focus of future work.

In addition to pore size, the 1.4 mm spacing between pores was chosen from previous research showing cells infiltrate approximately 1 mm into a meniscal tissue scaffold\textsuperscript{117–119}. Therefore, it was assumed that the diagonal spacing between pores of 2 mm should allow cells to infiltrate the tissue in the future. However, this pore spacing has not yet been verified in terms of cell infiltration and will need to be fully studied in future work. Pore spacing should also consider that previous tissue engineering studies have focused on very porous synthetic polymers in hopes of providing space for
biological tissue to completely replace the synthetic material\textsuperscript{111}. In this study, native tissue already exists, thus the goal becomes maintenance of mechanical properties and repair of laser drilled holes, rather than creating larger spaces for new tissue growth.

Most mechanical testing of the meniscus has focused on pull to failure and stress relaxation testing, while testing during cyclic loading is less common\textsuperscript{24,37,88}. However, the meniscus is cyclically loaded during ambulation, making cyclic testing critical for the evaluation of the physiologic behavior of the tissue. Only the compressive hysteresis area was lowered after decellularization (Figure 4-5A); however, the compressive hysteresis area tended to increase after laser drilling (Figure 4-8A). Additionally, the peak stress of hysteresis was significantly lowered for radial tensile samples after decellularization (Figure 4-3C), but again, this value tended to increase after laser drilling (Figure 4-6C). Considering collagen fibers in the meniscus act as an elastic component, it is logical the hysteresis area would increase after laser drilling (as seen in compression and circumferential tension samples), since collagen fibers were partially interrupted and a portion of the elastic response is lost. However, in the case of radial tension samples, the hysteresis area tended to decrease after laser drilling which may be explained by the lack of elastic component to begin with (sample was not tested along the dominant alignment axis of the collagen fibers); thus, loss of overall material may explain the decrease. Importantly, it should be noted that in compression, the hysteresis area and most stress relaxation parameters were changed from native to decellularized samples, but these values tended to reverse or remain unchanged after laser drilling. It should also be noted that the reduced sample numbers in native versus decellularized tensile hysteresis testing (from \(n = 12\) to \(n = 5–6\)) may have caused
potential effects to be missed. Power analysis predicted that the next closest nonsignificant parameter (radial hysteresis area) would be detected with 11 samples. However, for other nonsignificant parameters, sample numbers would need to almost double or be well above 50.

The SDS decellularization method has been previously shown to remove cells while maintaining mechanical properties\textsuperscript{94,116,125}, and histology confirmed decellularization in this study (Figure 4-2). Decellularization results from this study agree with those of Stapleton et al. who saw no significant differences between native and decellularized menisci in terms of tensile Young’s modulus, UTS, and failure strain\textsuperscript{94}. Stapleton et al. also compared native and decellularized menisci through indentation testing and saw no significant differences for 1–2 N loads\textsuperscript{94}. However, this study found a decrease in modulus after decellularization, while other studies found a decellularized meniscus to be 17% stiffer than native tissue\textsuperscript{18,37}. We also found significant changes in the stress relaxation parameters between native and decellularized tissues, which have not been reported in previous studies. These changes in stress relaxation values may be caused by the SDS interrupting noncovalent bonds in the extracellular matrix\textsuperscript{113}.

In a follow-up study, a dimethylmethylene blue (DMMB) assay was completed to measure glycosaminoglycan (GAG) content on a separate cohort of native and decellularized meniscus samples (n = 6 each). The DMMB assay showed GAG content was reduced from 1.95% (by weight) for native tissue to 0.02% for decellularized tissue. The loss of GAG content is to be expected with SDS treatment\textsuperscript{94,126}; thus, it should be considered that some changes in mechanical properties are related to GAG loss rather than cell loss. GAGs are charged molecules which create osmotic swelling and
compressive stiffness in native menisci\textsuperscript{61,94,127}. Thus, the changes in compressive properties after decellularization may be attributed to GAG loss. Additionally, since GAG loss can reduce water retention, changes in geometry between native and decellularized samples are expected. In this study, native compression samples had a statistically larger height and diameter compared to their decellularized counterpart sample (paired t-test, $p = 0.013$ for height and $p = 0.011$ for diameter). There was no difference in geometry between decellularized and laser drilled samples (paired t-test, $p \geq 0.068$), likely because these groups experienced the same GAG loss through processing.

While changes were seen in the stress relaxation parameters after decellularization, most changes reversed after laser drilling, much like the previously described return of hysteresis loading after laser drilling. There was a significant decrease in the SLS derived steady-state modulus and a significant rise in the QLV derived elastic parameter A and the viscoelastic parameters b and d after decellularization, but these values tended to reverse after laser drilling (Tables 4-1 and 4-2). Juran et al. hypothesized that improvement of TMJ disk mechanical properties after laser drilling may be due to improved rehydration of the tissue after lyophilization, which allows water molecules to recreate noncovalent bonds disrupted by SDS and partly restore mechanical properties\textsuperscript{113,128}. Given the similarities of the TMJ disk and the knee meniscus, these effects could also be occurring in the knee meniscus. It should also be noted that some differences were observed between the two decellularization groups which may be explained by different lots of animal tissue being purchased several months apart from animals of unknown age, breed, or sex. Unfortunately, this
information was not obtainable from the supplier. Regardless, this experiment used a paired design; thus, changes after decellularization and laser drilling were compared to a sample from the same meniscus. This design allows us to evaluate the effects of decellularization and laser drilling despite significant variability between menisci.

It should also be noted that the varying storage times in protease inhibitor may have introduced additional sample variability. However, the effects of this storage on mechanical properties are currently unknown.

**Conclusions for the Decellularizing and Laser Drilling the Knee Meniscus**

Our data, when combined with prior reports of reseeding acellular fibrocartilaginous tissues\textsuperscript{113}, demonstrate a potential to engineer an acellular porous native meniscus replacement which can withstand physiologic loading while integrating with the native environment. While the anticipated reductions of mechanical integrity were observed, these changes remained near physiologic values, indicating optimization of the laser drilling pattern and/or reseeding the decellularized tissue with cells may yield a viable meniscal replacement in the future. Furthermore, while reduced, many parameters remained well above the approximated in vivo stress values. Combined with prior work that indicates increasing the porosity of meniscus and temporomandibular disk scaffolds allows for improved fibrous ingrowth and cellular infiltration\textsuperscript{109–113}, our data indicate the porosity of decellularized meniscal allografts can be increased without a significant reduction in mechanical properties, thereby retaining sufficient mechanical integrity while encouraging fibrocartilaginous and vascular ingrowth for long-term health.
Conclusions for Part 1

Chapters 2, 3, and 4 have provided the current state of tissue mechanics for the knee meniscus, including the need for accurate testing to validate tissue engineered constructs. Testing the effects of hydrating the meniscus in synovial fluid versus saline revealed no differences in tension or compression testing; however, compression testing in synovial fluid was found to lower data variance. Then, decellularization and laser drilling were evaluated as a first step for engineering a meniscus replacement. Here, laser drilling statistically lowered some mechanical properties, but these properties largely remained in a physiologic viable range. Thus, it may be possible to create a meniscus allograft which maintains most of the complex fiber architectures and loading capabilities of native tissue, while also encouraging integration with the joint for long term survival.

Part 2 will transition into gait mechanics and how gait analysis may be used to quantify changes related to osteoarthritis in preclinical animal models. The following chapter begins with an introduction to osteoarthritis, while the following chapter will introduce gait analysis methods in osteoarthritis models.
CHAPTER 5
INTRODUCTION TO PART 2: DEFINING OSTEOARTHRITIS AND REVIEWING RESEARCH METHODS

Defining Osteoarthritis

Historically, osteoarthritis was thought to arise from “wear and tear” which deteriorated articular cartilage over time, as described in the following quotations from 1952 and 1972, respectively:

“Osteoarthritis of the hips, knees and spine develops from undue strains of excessive weight-bearing as life advances.”129

“Osteoarthritis is a non-inflammatory disorder of movable joints characterized by deterioration and abrasion of articular cartilage, and also by formation of new bone at the joint surfaces.”130

The above references demonstrate the historical perspective that osteoarthritis is purely a mechanical disease, in which too much load is placed on cartilage causing it to deteriorate. In accordance with these definitions, clinicians began diagnosing osteoarthritis with radiographs in the 1950s, looking at joint space narrowing as an indicator that articular cartilage was no longer present. Patients were likely instructed to “take it easy” and try to preserve their remaining cartilage.

As populous generations began to age, osteoarthritis came to the forefront of orthopaedic research. It is now understood that osteoarthritis degeneration is caused by a combination of genetics, weight, inflammation, injury, and/or metabolism. The revival of viewing osteoarthritis as a disease which affects the whole joint may be linked to Loeser et. al in 2012. This seminal position paper describes osteoarthritis as a “disease of the joint as an organ”131, and it is now accepted that osteoarthritis is not only defined as joint degeneration due to mechanics or overuse, but rather as an
encompassing disease with pathogenesis caused by injury, joint dysfunction, inflammation, metabolism, and catabolic activity.

**Osteoarthritic Changes throughout the Joint**

Along the mindset of osteoarthritis being a disease of the joint, one must view osteoarthritic changes at varying levels of the joint, much like multiscale biomechanics looks at different levels of mechanics. The following section will review osteoarthritic changes from the cellular level up to patient symptoms.

At the cellular level, chondrocytes become increasingly senescent with age, meaning the cells no longer divide and replace each other\(^\text{132}\). The transition from active to senescent chondrocytes may contribute to imbalance in the joint environment, as is associated with osteoarthritis. This cellular response to osteoarthritis can be sectioned into a biosynthetic and degradative phase. In the biosynthetic phase, articular cartilage chondrocytes attempt to repair damage, while enzymes begin to digest cartilage in the degradative phase\(^\text{133}\). Thus, a positive feedback loop is initiated where chondrocytes attempt to repair damage, but are outweighed by mediators which induce cartilage loss.

These changes at the cellular level often lead to a state of maladaptive repair in the joint (Figure 5-1, next page). While osteoarthritis is typically thought of as a catabolic event leading to cartilage degeneration, the cellular feedback loop previously described actually results in several anabolic responses in an attempt to repair the joint. These anabolic changes include the synovial lining becoming thick and fibrotic, osteophyte formation, subchondral bone sclerosis, and cartilage thickening in some areas.
Maladaptive repair is shown in a degenerated joint stained with toluidine blue. Although osteoarthritis is typically associated with catabolic (C) activity in the cartilage, there are many anabolic (A) repair responses occurring simultaneously. Photo courtesy of the Orthopaedic Biomedical Engineering Laboratory at the University of Florida.

Biochemical changes during osteoarthritis may also be studied in joint tissues and other bodily fluids. Senescence at the cell level has been associated with increased intercellular signaling of up to 80 growth factors, proteases, chemokines, and cytokines which have collectively been termed a senescence-associated secretory phenotype (SASP)\(^{134-136}\). A majority of SASP factors stimulate inflammation, such as IL-1, IL-6, IL-8, MCP-1, MCP-2, and MMP-1\(^{137}\). It has been hypothesized that the associated inflammation from SASP factors play a role in osteoarthritis development by putting the joint into a chronic “injury state”.

Perhaps the most common indicators of osteoarthritis are structural changes in the joint, and much research has been conducted on the structural changes in articular cartilage, subchondral bone, meniscus, and synovium. Early cartilage changes predating osteoarthritis include increased tissue hydration\(^{138}\), collagen transformation into a disorganized network\(^{139}\), and proteoglycan breakdown\(^{140,141}\). These changes
contribute to chondrocyte death, fibrillation in the articular cartilage, and eventual breakdown of cartilage. Changes in bone structure include subchondral sclerosis which reduces the bone’s ability to absorb a mechanical load and causes increased cartilage deformations\textsuperscript{142,143}. In addition to subchondral sclerosis, cartilage outgrowths may form on bone and calcify into osteophytes. Menisci from osteoarthritic joints have increased water content, deteriorated extracellular matrix, altered cell distribution, and altered collagen distribution\textsuperscript{144}. Lastly, synovial lining often becomes inflamed and may correlate with pain symptoms\textsuperscript{145}. Synovial cells also become more dense, but less aligned with the synovial edge\textsuperscript{146}.

In addition to joint structure, studies have been undertaken as to how these structures respond to altered biomechanics during osteoarthritis progression\textsuperscript{147–151}. Research in this field is relatively mature, as there are known relationships between joint mechanics and osteoarthritis development. For example, research indicates that a traumatic injury to the joint will alter the biomechanics and increase osteoarthritis risk 4-fold\textsuperscript{99,100,11,101}. Additionally, an increased knee adduction moment increases the risk for medial compartment osteoarthritis\textsuperscript{152,153}, but toeing-in can laterally shift the center of pressure to decrease the knee adduction moment\textsuperscript{150,154}. Furthermore, increased knee flexion excursion is associated with increased vertical ground reaction forces, while increased knee flexion angles are correlated with higher loading in the sagittal plane\textsuperscript{150,155}. However, whether mechanical changes are a compensation for tissue degeneration and pain or the cause of degeneration remains unanswered\textsuperscript{156}. Moreover, relationships between mechanics and pathology may evolve as osteoarthritis progresses. Unfortunately, studying the contributions of these factors in osteoarthritis
patients is highly complex due to multifaceted and often unknown etiologies of osteoarthritis.

While joint structure and function are typically used to diagnose osteoarthritis, the patient's focus is usually the pain experienced. This becomes complicated for clinicians and researchers because there is a discordance between patient symptoms and radiographic evidence of osteoarthritis\textsuperscript{157,158}. Patients typically report knee osteoarthritis pain as generalized or focused on the medial side\textsuperscript{159}. While some correlations have been found between pain scores and evidence of osteoarthritis, these scores are subject to bias and biopsychosocial factors. Pain may be associated with synovitis, subchondral bone changes, general inflammation, and/or neuropathy.

While these levels of osteoarthritis research and focus are all critical, researchers must begin to understand how these levels interact with one another to result in pain and disability.

**Clinical and Preclinical Disconnect in Osteoarthritis Research**

Clinical research focuses on patient symptoms and understanding human biomechanics to reduce joint loading and pain. While this strategy addresses the main patient complaint of pain and dysfunction, it does not aim to understand why the patient developed osteoarthritis and how to prevent or reverse the disease. Preclinical research traditionally looks at the other end of the spectrum by studying the joint with histology and biochemical analyses. However, these strategies struggle in translation to the clinic due to cross species differences and the inability to perform direct, and often invasive, pathophysiology measures in humans. Thus, in recent years, an attempt has been made to combine these research philosophies by studying behavior and
mechanics in preclinical models. However, a major challenge of osteoarthritis research lies in understanding the complex interplay between symptoms and pathology, and these occur across different levels of the joint during osteoarthritis progression.

Preclinical Models for Osteoarthritis Research

Preclinical osteoarthritis models may be classified as spontaneous or induced. In spontaneous osteoarthritis models, animals that are predisposed to arthritis development are used, such as the Hartley guinea pig. Transgenic models lie somewhere between spontaneous and induced osteoarthritis models, where an animal’s genome is altered to predispose the animal to osteoarthritis. Induced osteoarthritis models are most common and include intra-articular injections, joint immobilization or overloading, and surgical alteration.

Intra-articular injections of enzymes, growth factors, chemicals, and other substances are relatively simple to administer, but may not truly mimic osteoarthritis pathogenesis. For example, injection of collagenase will actively degrade cartilage; however, this model is also associated with severe inflammation, above what is normally present in osteoarthritis. Similarly, intra-articular injection of monoiodoacetate (MIA) has been used extensively in preclinical models, especially relating to pain. MIA inhibits glycolytic pathways, leading to widespread cell death, subchondral bone necrosis, and prolonged inflammation. Again, while the MIA model is relatively simple to administer and causes rapid changes, these changes are not typical of osteoarthritis, especially when comparing to the human condition.

Another strategy for osteoarthritis induction is to immobilize the joint to cause atrophy and cartilage erosion. However, immobilization-associated osteoarthritis is a
relatively small patient subset, and even in small animals, this type of cellular atrophy is not indicative of other osteoarthritis phenotypes. Rather than immobilizing the joint, others induce osteoarthritis by overloading the joint to directly injure cartilage. Overloading models are likely more representative of post-traumatic osteoarthritis. Moreover, overloading can be used to induce a noninvasive ACL rupture in mice\textsuperscript{177}. While this, non-invasive anterior cruciate ligament tear model is rapidly gaining popularity in osteoarthritis research groups, this model is still relatively under-studied and is somewhat less repeatable than surgical models at this time.

Most commonly, surgical methods are used to induce preclinical osteoarthritis. With surgical transection of ligaments or the meniscus, joint loading and mechanics will be altered, in addition to inducing inflammation. Changes in joint histology following surgical induction of osteoarthritis most closely resemble human pathology, and similar to traumatic injuries in humans, surgical models have a relatively low progression of joint degeneration following injury, allowing researchers to methodically plan experiments to study OA progression temporally. However, as no model is perfect, surgical models require incisions and associated soft tissue damage that is again atypical of joint trauma\textsuperscript{178}. Moreover, separating post-surgical pain from osteoarthritic pain can be challenging.

\textbf{Analysis of Preclinical Rodent OA Models}

While it is possible to study interactions between gait mechanics, cartilage biology, and osteoarthritis mediators in large animal models, these studies are complicated by expense and a relative under-development of biological assays. Rat
models overcome many biological limitations, while providing an opportunity to evaluate OA symptoms through behavioral analyses.

The most common assessment of joint structure in humans and large animals is radiographs; however, radiographs only provides information about bone and relative joint spacing. For more detail, an MRI may be performed to view soft tissue in the joint. Additionally, computed tomography machines have advanced to resolutions on the micro-scale, which may be useful for small animal models. In particular, EPIC (equilibrium partitioning of an ionic contrast agent) CT may be used to image morphology of cartilage and other soft tissues by soaking the sample in specific contrast agents\textsuperscript{179}. However, these methods are costly for preclinical research and are still being validated for rodent models.

A more detailed joint assessment is achieved through histology. Histology provides a view of the entire joint, and may be stained for various proteins using immunohistochemistry. Additionally, histology is commonly classified using standard grading schemes, such as those provided through OARSI\textsuperscript{180}. However, grading schemes are often only semi-quantitative and may vary among laboratories and graders. Additionally, histology requires euthanasia and thus can only be performed at single time points.

Another option to quantify osteoarthritis involves measuring biomarkers in the blood, sera, urine, and synovial fluid. Biomarkers provide a quantitative measure that describes the catabolic effects of osteoarthritis progression and can be measured temporally in the same animal. However, biomarker collection in synovial fluid (where most joint-level markers are expected to be at a higher concentration) is very difficult in
small animals due to small volumes. Biomarker levels in the blood and serum have been quantified and associated to osteoarthritis\textsuperscript{181,182}; however, biomarker detection is not yet possible in the early stages of disease progression, where treatment may be most effective. Given osteoarthritis is driven by a local catabolic and pro-inflammatory environment\textsuperscript{131}, joint-level biomarkers are likely more sensitive than blood and serum levels\textsuperscript{183}.

Finally, preclinical osteoarthritis models may also be analyzed using behavioral tests. For mechanical sensitivity, von Frey filaments are commonly used in association with the Chaplan up down protocol\textsuperscript{184}. While this test is fairly easy to administer and may be conducted temporally in the same animal, von Frey testing is subject to researcher bias. Moreover, the test is typically administered in the paw, which only reflects referred sensitivity from the joint. As such, the results may be most associated with supraspinal reflexes and not nociceptive changes in the joint itself. Other behavioral tests include activity monitoring and qualitative scoring\textsuperscript{185}. These methods require video to study the animal’s behavior, but often the researcher is left to assign scores on movement and behavior. The incapacitance meter records weight bearing on the hind limbs as the animal is confined in an upright position. While this measure is quantitative and provides information on limb loading, the test requires animal confinement and stress which may skew results. Similarly, the rotarod measures how long an animal can walk on a rotating rod without falling off. While this test may correlate to balance, it may also be influenced by animal stress, distractions, and research environment.
Operant behavioral tests are designed such that the animal is not forced to participate in a test, and instead aims to record natural movement and responses. Behavioral tests which are operant-based and stress-free are ideal for preclinical studies. While a few of the above mentioned tests are stress-free, such as activity monitoring, these tests are rarely quantitative, which limits the sensitivity of the measure. Thus, gait analysis may be used to quantify behavioral changes in musculoskeletal models. There are several methods of collecting rodent gait, but the main outcomes are spatiotemporal gait parameters and ground reaction forces. Commercially available gait systems include CatWalk, DigiGait, and TredScan, which output spatiotemporal parameters and paw areas, but do not include direct measures of ground reaction forces. Additionally, treadmill based gait systems, such as the DigiGait, are not operant, induce animal stress, and may mask gait changes\textsuperscript{186,187}. Custom gait recording methods have been able to collect robust spatiotemporal gait parameters via high speed cameras while simultaneously recording ground reaction forces\textsuperscript{188–190}. The following chapter includes an in depth review of preclinical gait analysis.

Part 2 of this dissertation will examine two preclinical models of osteoarthritis. First, a low dose of monoiodoacetate will be used to induce low grade joint damage, and quadrupedal gait analysis will be used to evaluate the effect of this chemical. Secondly, destabilization of the medial meniscus will be used in mice, and quadrupedal gait analysis will be use to discern how male and female mice adapt to this injury. Thus, part 2 will provide a thorough analysis of rodent gait for osteoarthritis models.
Usefulness of Gait Analysis in Rodents

Technological advances have made gait analysis a widely available tool for rodent models; however, gait analysis, itself, is not a new practice. Aristotle wrote on human motion, and scientists such as Boerhaave, Euler, and Carlet advanced the understanding of gait mechanics throughout the eighteenth and nineteenth centuries. In the late 1800s, human and quadrupedal gait patterns were famously recorded by Muybridge, and Hildebrand plots have been standard descriptors of the temporal gait sequence of quadrupeds since the 1960s\(^1\).\(^2\) In recent years, gait technologies have continued to improve, and with advances in high-speed videography and force plates, gait is now possible in smaller organisms, including preclinical arthritis models.

In the clinical assessment of arthritis, gait analysis can be effectively used to assess mobility and function, and while the quadrupedal gait patterns used by rodents clearly vary from bipedal human patterns, the conceptual basis for gait analysis remains analogous - an altered gait pattern can be used to protect an injured limb from loading and/or movement-evoked pain. As such, several compensatory patterns are shared between quadrupeds and bipeds, including shuffle-stepping and limping\(^1\).\(^8\). The frequent challenge in rodent gait analysis is that these compensatory patterns can be difficult to detect due to the ability to re-distribute load to three limbs rather than one and the rapidity of the gait sequence. Thus, although rodent gait varies significantly from

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human gait, it remains important to advance technologies for rodent and quadrupedal gait analysis.

In addition, bench to bedside translation in arthritis historically begins by testing new therapies in rodents before translating to larger organisms and clinical studies. However, gait analysis has followed the opposite trend, with sophisticated motion tracking and force measurements first being developed for humans and then scaled down to large animals and rodents. This trend is driven by scale advantages, with gait compensations being easier to detect in humans and large animals relative to rodents. In addition, rodents are prey animals, and evolution has likely conditioned rodents to mask signs of disability and pain\textsuperscript{193–196}, making rodent gait compensations relatively more difficult to detect. Only recently have sophisticated gait tracking systems been applied to rodent arthritis models\textsuperscript{188,190,197–199}, and even with these approaches, gait parameters are typically limited to the spatiotemporal pattern. Thus, even though quadrupedal gait has been studied for decades, sophisticated rodent gait analyses have only recently been applied to preclinical arthritis models. Nonetheless, use of gait analysis to study preclinical arthritis models will likely continue to expand.

In rodent models, gait is classified as a behavioral analysis. In her book, What's Wrong With My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice, Jacqueline N. Crawley, Ph.D. takes care to inform scientists of the complexity of rodent behavioral analyses, stating:

“As in any field of science, behavioral research has evolved proper experimental designs and controls that must be correctly applied for the data to be interpretable. Little things, such as how to handle the mouse to reduce stress, can greatly affect the results of a behavioral experiment. Like microinjecting an oocyte or operating a DNA sequencer, the tricks of
the trade are best learned from experts. You don’t want to waste your time reinventing the wheel."

Professor Crawley’s recommendation rings true for quadrupedal gait analysis as well. While the use of gait to assess rodent arthritis models is a relatively modern concept, the study of quadrupedal gait is not. Unfortunately, most commercial rodent gait analysis systems inundate users with large datasets - sometimes 50+ variables - to describe the rodent spatiotemporal gait pattern. Moreover, most of these variables are not independent and can be difficult to interpret without an understanding of quadrupedal gait. The goal of this review is to describe the state of rodent gait analysis and highlight fundamental gait parameters needed to accurately evaluate preclinical arthritis models. Rodent gait parameters include observational scores or measurements of spatiotemporal, kinetic, and kinematic gait characteristics. We structured this review with these sections, describing research conducted in these areas and the strengths and limitations of current approaches (see Table 5-1 at the end of this chapter for introductory terminology).

Observational Scoring

Terminology

Observational scoring uses rank-order scales to grade the severity of rodent gait abnormalities (Table 6-2, end of chapter). Since these scales have been developed by individual labs, scoring systems are relatively inconsistent, with some defining 0 as normal while others define the maximum as normal. Thus, when comparing results across laboratories, it is important to first understand the scale.
Review of Observational Scoring Techniques in Rodents

Visually scoring an animal's gait may be the simplest form of gait analysis. Scoring systems often seek to identify “guarding gaits”\textsuperscript{202,203}, while others report a similar non-specific gait score that characterizes limping or failure to fully apply weight\textsuperscript{204–208}. Advantages of scoring include individualized scales relevant to particular disease models, high throughput, and relatively simple data analysis. However, scoring systems are subject to observer bias and typically provide only semi-quantitative gait descriptions.

Recommendations for Gait Scoring in Rodents

Observational scoring is typically performed by placing the animal in an open arena and directly scoring the animal or recording video to be scored later. Due to the rapidity of rodent gait, the human eye is often unable to detect subtle changes without assistance from videography, and these subtle gait compensations may be more likely to associate with diseases like osteoarthritis\textsuperscript{185,187,193,209}. Hence, we recommend video be used for observational scoring whenever possible. In addition, since scoring is largely an assessment of self-selected behaviors, open arena testing is ideal to capture the animal's natural gait. Some laboratories have used treadmills and food incentive for these tests, but these factors could mask some gait characteristics\textsuperscript{187}. Finally, as with any behavioral analysis, investigators should learn proper techniques to handle and acclimate their animals to the experiment.

For studies where gait is not a primary outcome measure, observational scores are a quick, though insensitive, measure of rodent behavior. In arthritis models, these measures could be improved by generating standard scales, similar to the BBB score in
spinal cord injury\textsuperscript{185}. But, this too is inhibited by the broad range of severities observed in arthritis models. As a general recommendation, observational scoring using videography should not use terms such as “mild” or “severe,” as these terms are open to interpretation. Instead, scoring should be distinguished by tangible occurrences, such as the “guarding after noxious compression” definition used by Boettger et al\textsuperscript{185,202}. Similarly, without videography, scoring should use a measurable system to categorize animal behavior, such as the ink prints used by Kumar et al\textsuperscript{208}. Nonetheless, there is a need to refine these scales, but even with standardization, scientists should recognize observational methods will only provide a generic and relatively insensitive measure of rodent gait compensations.

**Spatiotemporal Characterization**

**Terminology**

Spatiotemporal gait parameters describe how an animal’s paws move in both space and time, including stride length, step width, duty factor, temporal symmetry, limb phase, and characteristics of the footprint (Figure 6-1). Spatial parameters are generally familiar variables (Figure 6-1a). Some gait systems also report variables such as the distance and angle between paws, but scientists should recognize these are not independent measures and can be geometrically derived from stride length, step length, stride length asymmetry, and step width\textsuperscript{187}. In addition, novice users should remember the difference between step length and stride length; while the left and right step length can differ (spatially asymmetric), the stride length must be the same for all limbs if the animal is using a consistent gait pattern. In addition to these classic spatial descriptors,
the orientation and area of each footprint can be analyzed, as well as the relative position of the toes\textsuperscript{210,211}.

Figure 6-1. Spatiotemporal gait parameters. A) Spatial gait parameters are shown for the rat hind limbs. Fore limbs prints were removed for clarity. B) Temporal gait parameters are described with a Hildebrand plot for quadrupedal rodent gait. Dashed lines indicate a moment in time in the gait cycle, which is depicted by the rat drawing above each line.
Temporal gait parameters are typically less familiar to the non-specialist. The classic parameters of the quadruped temporal gait sequence derive from the seminal work of Milton Hidebrand\(^{192}\) (Figure 6-1B). These parameters include duty factor, defined as the ratio of the limb stance time and limb stride time\(^{187,199,212}\).

\[
\text{Limb Duty Factor} = \frac{\text{stance time of limb}}{\text{stride time of limb}} \tag{6-1}
\]

To make the nomenclature more obvious to the non-specialist, our laboratory and others have referred to limb duty factor as percentage stance time, but these variables are identical. Temporal symmetry is defined as the time between a right foot-strike and left foot-strike divided by the stride time\(^{187,212}\).

\[
\text{Temporal symmetry} = \frac{(\text{time of right foot strike} - \text{time of left foot strike})}{\text{stride time}} \tag{6-2}
\]

Finally, limb phase is defined as the time between forelimb and hind limb foot-strike on the same side divided by the stride time. Please note, for a gait sequence to be repeatable, the animal must have approximately the same stride length and stride time on all four limbs.

\[
\text{Limb phase} = \frac{(\text{time of left fore foot strike} - \text{time of left hind foot strike})}{\text{stride time}} \tag{6-3}
\]

Review of Spatiotemporal Gait Analysis Techniques for Rodents

A summary of papers utilizing spatiotemporal analyses is provided in Table 6-3 (end of chapter). Early spatial pattern descriptions were analyzed by having the animal walk across an ink pad followed by a sheet of paper and measuring the spatial pattern of the ink prints. Using this technique, rats with antigen induced arthritis had asymmetric spatial patterns indicated by decreased step lengths, and increased paw angles in arthritic rats\(^{202}\). As discussed below, a major limitation of ink pad methods is the inability
to accurately measure animal velocity, a critical covariate for nearly all gait parameters. In addition, the amount of ink is inconsistent between steps and trials, thus paw print areas are highly variable. As such, modern high-speed videography is more likely to provide a robust analysis of spatial parameters.

The CatWalk (Noldus) measures spatiotemporal characteristics along with paw print intensity. In the CatWalk, a light is shined into the side of a glass walkway. When the animal makes contact with the glass, light reflects downward and is captured by a camera beneath the floor (typically recording at 100-150 fps). Advantages of this method include illuminating the portion of the foot touching the floor. The CatWalk calculates 25 parameters based on footprints and 10 parameters based on time, and for arthritis studies, footprint intensities and areas are the most commonly reported parameters\textsuperscript{199,213–216}. As an example, mice with CFA-induced ankle arthritis had less regular gait with changes in speed and duty factor\textsuperscript{199}.

The DigiGait (Mouse Specifics, Inc.) uses a clear treadmill with a camera underneath to record foot-strikes at 125 fps. DigiGait reports over 50 gait indices, with the parameters most commonly reported including stride length, stance time, swing time, paw area, and braking/propulsion times. On the DigiGait, mice with collagen induced arthritis showed increased stride frequency and paw area and decreased stride length, stride time, paw angle, stance time, and swing time\textsuperscript{217}.

CleverSys offers the GaitScan, which uses a similar approach to the CatWalk, and the TreadScan, which is a treadmill system similar to the DigiGait. A main difference between the TreadScan and DigiGait is the camera (100 fps) positioning; on the Treadscan, a 45° mirror positioned below the treadmill allows a single camera to
capture both lateral and ventral views of the animal. Using the TreadScan, rats with adjuvant induced arthritis had a reduction in stride length and increase in stance time and swing time\textsuperscript{218}.

Even though DigiGait and TreadScan work on similar principles, these systems do not necessarily achieve the same results. In a rat model of carrageenan-induced arthritis, the five parameters relating to the injured foot had opposite results when tested on the DigiGait vs TreadScan\textsuperscript{219}. The authors suggest differences in chamber size, color, lighting, and treadmill belts may explain the variation; however, neither system provided acceptable reproducibility\textsuperscript{219}. This highlights the importance of consistency and is a reminder that small methodological factors can play a large role in rodent gait analysis.

Finally, custom gait analysis systems have been developed by several laboratories. Our lab has constructed an open arena walkway, which allows the animal free mobility with no stressors or rewards. Using a mirror oriented at 45° under the floor, we use a high-speed camera (recording at 250 to 1,000 fps) to capture lateral and ventral views of the animal. Having these views allows foot-strike and toe-off (temporal variables) to be measured in the lateral plane and the foot position (spatial variables) to be measured in the ventral plane. Moreover, this platform has allowed us to detect subtle spatiotemporal gait changes, such as spatial and temporal symmetry, duty factor, single-limb support, and temporal shifts of 0.001-0.025 s, in multiple rodent arthritis studies\textsuperscript{188,190,197,209}. 
Recommendations for Spatiotemporal Gait Characterization in Rodents

Many methods are available for rodent spatiotemporal gait analysis, and these systems have considerable advantages over the basic inkpad methods used a few decades ago. Unfortunately, the array of systems and overwhelming number of parameters reported often complicate the gait analysis. We recommend researchers use fundamental descriptors of the spatial pattern: stride length, step length, stride length asymmetry, and step width - and temporal parameters provided by Hildebrand - duty factor, temporal symmetry, stance time imbalance, and limb phase (when forelimbs are included). If available, measures of footprint size and orientation can also be useful.

An unfortunate trend has been the reporting of gait variables derived from the above parameters as “new” or “novel”. As an example, swing time ratio (swing time on one foot divided by the swing time on the opposite foot) has been presented as a new gait measure. However, when examining the Hildebrand plot, a change in swing time is associated with a change in stance time. Thus, swing time ratio measures the same gait change as stance time imbalance. This same shift has also been reported as a change in “single limb support”, a term common in bipedal human gait, but a bit of a misnomer for quadrupedal sequences. Regardless of the preferred nomenclature, the temporal shift is the same - not new.

In addition, investigators must also consider the effects of velocity. Stride length, step length, step width, and limb duty factor are highly correlated to walking speed; thus, accounting for velocity is absolutely essential. This can be done through statistical models, comparisons to controls, or by controlling velocity with treadmill. However, it should be noted treadmills can mask subtle spatiotemporal changes by inducing stress, and treadmill measurements vary
significantly from open arena measurements\textsuperscript{187}. Finally, in open arenas, investigators often provide “home cage” or food incentives. It is not known if these enticements alter rodent gait, since the animal may suppress limb dysfunction and/or pain to reach the reward. As such, we generally recommend open arena testing without an external stimulus, as these methods are most likely to obtain the self-selected gait pattern for each animal.

The camera recording speed can also affect the accuracy of spatiotemporal data. The Nyquist-Shannon rule states an effective sampling frequency should be greater than twice the duration of the fastest factor being measured. The magnitude of temporal shifts will depend on the severity of the arthritic condition. As such, compensations associated with inflammatory arthritis can often be detected at 100 fps. For milder forms of arthritis, our lab has shown frame rates above 200 fps are needed to detect compensatory gait patterns in rodents\textsuperscript{187}.

Finally, but most importantly, understanding how one spatiotemporal gait variable will affect other measures is critical to understanding the pattern. A single spatiotemporal parameter is unlikely to accurately describe subtle gait changes. As an example, both a decrease and increase in duty factor could indicate a gait compensation. For unilateral compensations, the affected limb duty factor decreases while the contralateral limb duty factor increases (limping, imbalanced gait sequence). However, for bilateral compensations, both hind limb duty factors increase (shuffle-stepping, balanced gait sequence). Both sequences can reduce limb loading; unfortunately, only unilateral compensations are typically considered in arthritis studies. However, we have observed bilateral compensations in a rat model of knee
osteoarthritis\textsuperscript{190}. Because of the complexity of quadrupedal compensations, it will be difficult to derive a single gait measurement that will capture the myriad of possible compensations. Thus, researchers must carefully consider how the gait pattern has changed both spatially and temporally and how this altered pattern may protect an injured limb.

**Kinetic Gait Parameters**

**Terminology**

Kinetics describes forces associated with movement. For rodents, kinetics are largely focused on dynamic weight bearing or occasionally on ground reaction forces (GRF)\textsuperscript{223,228–230}. For GRFs, the vertical component (z-axis) is the most commonly reported for rodent arthritis models. The anteroposterior force (x-axis) and the mediolateral force (y-axis) may also provide insights to rodent gait; however, the off axis forces (x, y) currently require highly-sensitive, custom platforms\textsuperscript{188,228,230}. In addition, the impulse on each axis (area under the force-time curve) is occasionally reported (Figure 6-2, next page).
Figure 6-2. Dynamic gait parameters. A) GRFs are shown for a rat foot with sign conventions. The mediolateral force points toward the midline of the animal, the braking/propulsion force points in the direction of travel, and the vertical force points upward. B) A standard ground reaction force curve is shown for all three force components.
Review of Kinetic Gait Analysis Techniques in Rodents

A summary of papers utilizing kinetics is provided in Table 6-4 (end of chapter).

The Dynamic Weight Bearing Test (Bioseb) uses an instrumented floor and video to calculate the percentage of weight on each leg. An advantage of this system is the animal is free to bear weight normally, unlike the incapacitance test. This test reports parameters including weight, surface area, and time spent on each paw, along with the variability of these measures. The Dynamic Weight Bearing Test has been used in medial meniscus transection and adjuvant induced arthritis studies, finding reduced weight placed on the injured limb in both models\(^{225,231,232}\).

The Pressure-Sensing Walkway (TekScan) uses an instrumented floor to measure spatiotemporal parameters along with paw pressure and impulse. This walkway has been used to study ACL transection in rats, finding increased hind limb maximum force ratios\(^{201}\). This system allows animals to walk freely. However, users should note the walkway measures foot pressures, which provides a measure of weight born on each limb, but cannot distinguish between the x, y, and z GRFs. Similarly, the CatWalk system (previously described) does not quantify limb forces, but does measure paw print intensity; and this parameter could be considered an indirect measure of limb loading.

Our lab has recently adapted our spatiotemporal arena to simultaneously record kinetic and spatiotemporal data. By instrumenting transparent sections of the floor with 3-axis force plates (Figure 6-3, next page), 3-component GRFs can be simultaneously determined with spatiotemporal gait parameters. However, this approach requires the animal to make contact with the instrumented floor panels, which markedly increases testing time.
Figure 6-3. Gait arena set-up. A) A craniocaudal view of our laboratory’s gait system is shown with high speed video being recorded from the side (lateral). Force plates are located outside the animal's path of travel so as to not obstruct view of the feet in the mirror underneath the floor. B) A lateral camera view of the arena showing force plate positioning and foot print visualization via the mirror.
Recommendations for Kinetic Gait Characterization in Rodents

Again, open arenas promote the most natural gait; however, these arenas may be non-ideal for kinetics due to the significant time associated with waiting for rodents to correctly contact the force panels. Treadmills have helped solve this problem in humans and larger animals\textsuperscript{186,233–235}, but creating a force-instrumented treadmill for rodents is difficult due to belt shear forces on the very sensitive and expensive force recording equipment. Moreover, to distinguish between right and left limb forces, human instrumented treadmills (AMTI) have split belts with two force plates, but walking on a split belt treadmill would be difficult to train in rodents. Interestingly, an instrumented running wheel has been developed to measure normal and tangential forces in rodents; however, to our knowledge, this system has not yet been used to study arthritis\textsuperscript{236}. Running wheels may provide an ideal environment for kinetic data collection, as rodents can use these wheels freely with the probability of a foot-strike on the instrumented rung greatly increased over an open arena.

Because so little is known about rodent GRFs, it is difficult to make strong recommendations on how to analyze these data. Clearly, additional studies and advanced methods are needed, and like spatiotemporal parameters, different compensation strategies may have different effects on kinetic variables (i.e., limping vs shuffle-stepping). In humans, foot center of pressure shifts laterally with medial knee arthritis\textsuperscript{237}, and the free moment increases after knee arthroplasty\textsuperscript{238}. However, it is not yet known whether these data are important or can be measured in rodents. Nonetheless, kinetics tends to be more descriptive of arthritic conditions in larger animals and humans, and as such, these methods may prove more sensitive in rodents.
Kinematic

Terminology

Kinematic parameters describe bony body positions and are commonly defined in terms of three joint angles and three translations (Figure 6-4). Knee kinematics may also be described as range of motion, which describes the minimum to maximum values for a rotation or translation; however, due to limitations in measuring knee kinematics in rodents, range of motion typically refers to flexion angles in the sagittal plane.

![Joint kinematics. Sagittal and craniocaudal views of a rodent leg are shown with three translations and three rotations indicated.](image)

Review of Current Methods of Kinematic Gait Analysis in Rodents

Kinematics are commonly studied in humans with skin markers; however, this technology does not scale to rodents due to excessive skin motion artifact\textsuperscript{239,240}. Nonetheless, skin-based motion tracking systems have been applied to rodents\textsuperscript{240–246}, but only ankle motion may be considered a reasonably accurate measurement of bone movement\textsuperscript{240}. Thus, while clearly valuable, knee, hip, and spine kinematics have not been fully characterized in rodent arthritis models.
Rodent kinematic techniques are developing, with several labs pursuing fluoroscopic techniques to track bone movements\textsuperscript{240}. In rats with antigen induced arthritis, sagittal fluoroscopy has detected knee flexion range of motion, with minimum joint angles of 40° in swing and maximum angles of 100° at end of stance\textsuperscript{203}. Similar studies recorded sagittal plane fluoroscopy in rodents as a proof of concept\textsuperscript{240,247}, and even these basic fluoroscopic methods greatly improved measurements relative to skin marker tracking. However, current rodent fluoroscopic methods measure 2D flexion angles from sagittal views, and in/out of plane joint positioning may skew these sagittal plane angles. Therefore, biplanar fluoroscopy, which has been used to solve this problem in humans, may also have advantages for rodent kinematics. Biplane fluoroscopy has been used in an evolutionary study of rodent gait\textsuperscript{248}, but to date, this technology has not been used to study rodent arthritis models.

**Recommendations for Kinematic Gait Characterization in Rodents**

Again, while open arena testing allows animals to walk naturally, a treadmill may be necessary to collect kinematic data, allowing the animal to stay in the field of view and control radiation doses during the collection of consecutive, repeatable gait cycles. Labs using fluoroscopy may also need to adapt their systems to collect at higher frame rates, since most machines only collect videos at 30 fps. As limb foot-strikes occurring approximately every 0.4 s in rats\textsuperscript{187}, this frame rate would only allow 12 images to be collected for a gait cycle.

Rodent fluoroscopy, to date, has largely used single plane, sagittal imaging to study the hind limbs. Single plane fluoroscopy allows small animals to be positioned closer to the fluoroscopy source to achieve image magnification, and single plane
fluoroscopy is more widely available across institutions. However, in other animals, biplane fluoroscopy has greatly improved accuracy and allowed 3D kinematic motions to be better studied. Moreover, metallic markers placed in the femur and tibia may allow more accurate tracking of skeletal motions \(^4,249,250\), though placing these markers may affect the rodent's gait. All of these techniques are currently in their infancy, but offer promise to improve our understanding of rodent arthritis models.

**Conclusions for Rodent Gait Recommendations**

The goal of this chapter was to review current technologies used in rodent gait analysis and provide recommendations for the use of rodent gait analysis in arthritis models. Through this review, we highlighted the relatively large, but current, literature available regarding spatiotemporal gait analysis. For kinetics and kinematics, the literature is more sparse, but viable technologies are clearly being developed. Additionally, though spatiotemporal parameters are the most widely used, several inconsistencies between testing systems and laboratories remain. Thus, rodent gait analysis has significant room for technological advancements in the coming years. When performing rodent gait analysis, it is important to remember that, while rodent gait analysis is a relatively modern concept, the study of quadrupedal gait is not new. Thus, even though most commercially available gait systems report multiple parameters, a balance needs to be found, where critical gait parameters are reported (excluding new ratios or geometric derivatives) while still considering gait changes as an entity. To reach this stage, rodent gait analysis should be advanced to accurately measure spatiotemporal, kinetic, and kinematic parameters, with consistency across laboratories.
As rodent gait technologies improve, preclinical models will be better understood and their utility for preclinical arthritis research will increase.
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spatial</td>
<td>Relating to positions in space</td>
</tr>
<tr>
<td>Temporal</td>
<td>Relating to time</td>
</tr>
<tr>
<td>Kinetic</td>
<td>Relating to the forces associated with motion</td>
</tr>
<tr>
<td>Kinematic</td>
<td>Relating to motion</td>
</tr>
<tr>
<td>Step Length</td>
<td>Distance from foot strike to subsequent foot strike of the opposite foot</td>
</tr>
<tr>
<td>Stride Length</td>
<td>Distance from foot strike to subsequent foot strike of the same foot</td>
</tr>
<tr>
<td>Step Width</td>
<td>Distance between the limbs perpendicular to the direction of travel</td>
</tr>
<tr>
<td>Duty Factor</td>
<td>Stance time divided by stride time</td>
</tr>
<tr>
<td>Temporal Symmetry</td>
<td>Time between a right and left foot-strike divided by stride time</td>
</tr>
<tr>
<td>Limb Phase</td>
<td>Time between same side forelimb and hind limb foot-strikes divided by stride time</td>
</tr>
<tr>
<td>fps</td>
<td>Frames per second</td>
</tr>
<tr>
<td>GRF</td>
<td>Ground reaction force</td>
</tr>
<tr>
<td>Impulse</td>
<td>Area under the force-time curve</td>
</tr>
<tr>
<td>Braking/Propulsion</td>
<td>Forces that occur in the direction of travel (also anteroposterior). Typically associated with slowing of the center of mass immediately after foot-strike (braking) and push-off forces propelling the center of mass forward immediately prior to toe-off (propulsion)</td>
</tr>
<tr>
<td>Mediolateral Forces</td>
<td>Forces that occur toward the animal’s midline. Typically associated with the stability of the animal and the left-to-right / right-to-left sway of the center of mass during gait</td>
</tr>
</tbody>
</table>
Table 6-2. Review of Observational Gait Scores Used in Rodent Knee Arthritis Models

<table>
<thead>
<tr>
<th>Species</th>
<th>Arthritis Model</th>
<th>Gait Method</th>
<th>Parameters Measured</th>
<th>Results</th>
<th>Therapies Tested</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>CFA</td>
<td>Score</td>
<td>0: Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1: Guarding after noxious compression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2: Visible limping</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>3: No use of the hind limb</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4: No movement at all</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mice</td>
<td>CFA</td>
<td>Score</td>
<td>0: Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carrageenan</td>
<td></td>
<td>1: Moderate impairment of stance, toes contracted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2: Severe impairment, foot elevated, toes together</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rats</td>
<td>CFA</td>
<td>Score</td>
<td>0: Normal</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>.5: Marked limping</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0: Three legged gait</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rats</td>
<td>Zymosan</td>
<td>Score</td>
<td>0: Normal</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1: Mild disability</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>2: Difficulty walking</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>3: Three legged gait</td>
<td></td>
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</tbody>
</table>

- Scores highest at day 1 and decreased over time. Still limping at day 21.
- Carrageenan: Highest scores 3 hrs after injection. CFA: highest scores 24 hrs after injection.
- Lame gait seen after injection
- Increased gait score after injection

Morphine, Dexamethasone
Diclofenac, Morphine
Diclofenac, Entada-phaseoloides
Naproxen, ATB-346

203
207
205
206
<table>
<thead>
<tr>
<th>Species</th>
<th>Arthritis Model</th>
<th>Parameters Measured</th>
<th>Results</th>
<th>Therapies Tested</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>Collagenase</td>
<td>Score</td>
<td>Decreased score after injection</td>
<td>BoNT/B</td>
<td>204</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4: Normal</td>
<td>3: Minimal impairment</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2: Moderate impairment</td>
<td>1: Significant impairment</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0: Won’t walk on treadmill</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Collagen Type II Antigen</td>
<td>Score</td>
<td>Increased guarding scores up to day 14. Scores returned to control levels on day 21.</td>
<td>Enbrel (anti-TNF)</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0: Normal</td>
<td>1: Guarding after noxious compression</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>2: Visible limping</td>
<td>3: No use of the hind limb</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4: No movement at all</td>
<td></td>
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</tr>
<tr>
<td>Rat</td>
<td>PGPS</td>
<td>Ink Prints</td>
<td>High scores (3.5) after induction of arthritis.</td>
<td>FX006 with TCA IR</td>
<td>208</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0: Normal</td>
<td>1: Slight limp, mainly toe prints on injured foot</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2: Limping, only toes</td>
<td></td>
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<tr>
<td></td>
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<td>3: Dragging and carrying leg, drag marks</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>4: Carrying leg entire time, no staining from injured foot</td>
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</tbody>
</table>

CFA: Complete Freund’s Adjuvant; PGPS: Peptidoglycan Polysaccharide
<table>
<thead>
<tr>
<th>Species</th>
<th>Joint</th>
<th>Arthritis Model</th>
<th>Gait Method</th>
<th>Parameters Measured</th>
<th>Results</th>
<th>Therapies Tested</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>Ankle</td>
<td>CFA</td>
<td>CatWalk</td>
<td>Inter-limb coordination, stance phase, swing phase, duty factor, stride length, and swing speed</td>
<td>Less regular gait, changes in stance phase duration, swing phase duration, speed, and duty factor. Reduced ratio of right to left hind limbs for paw pressure, print area, stance phase duration, duty factor, and swing speed.</td>
<td>Indomethacin</td>
<td>199</td>
</tr>
<tr>
<td>Rats</td>
<td>Knee</td>
<td>MIA or ACLT</td>
<td>CatWalk</td>
<td>Swing time, swing speed, and duty factor. Velocity as time to cross arena/length of arena</td>
<td>No changes in velocity for any group. MIA: Longer swing phase, slower swing speed, and smaller duty factor in ipsilateral limb. ACLT: No difference in swing phase and swing speed between limbs. Changes in both limbs for swing phase and greater speed compared to controls.</td>
<td>Celecoxib</td>
<td>251</td>
</tr>
<tr>
<td>Mice</td>
<td>Ankle</td>
<td>LPS</td>
<td>CatWalk</td>
<td>Paw pressure intensity, print area</td>
<td>Ratio of right/left hind paw pressures and the ratio of right/left hind paw areas were lowered after 2 days.</td>
<td>Indomethacin, Minocycline</td>
<td>213</td>
</tr>
<tr>
<td>Rats</td>
<td>Knee</td>
<td>Type II Collagenase</td>
<td>CatWalk</td>
<td>Paw print intensity</td>
<td>Reduced paw intensity in collagenase injected rats.</td>
<td>Morphine, Lidocaine, Diclofenac</td>
<td>214</td>
</tr>
<tr>
<td>Species</td>
<td>Joint</td>
<td>Arthritis Model</td>
<td>Gait Method</td>
<td>Parameters Measured</td>
<td>Results</td>
<td>Therapies Tested</td>
<td>Citation</td>
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</tr>
<tr>
<td>Rats</td>
<td>Knee</td>
<td>MIA</td>
<td>CatWalk</td>
<td>Paw print intensity and area, velocity, stride length, stance time, stride time, swing time</td>
<td>Decreased paw print intensity. None No change in other parameters.</td>
<td></td>
<td>252</td>
</tr>
<tr>
<td>Rat</td>
<td>Knee</td>
<td>ACLT</td>
<td>CatWalk</td>
<td>Velocity, stance duration, swing duration. Calculated a “limb idleness index” from paw print intensity ratios.</td>
<td>Increased LII, target print ratio, and swing duration ratio. Unchanged anchor point ratio. Comparing sham and naive, there was higher LII, but no difference in ratios.</td>
<td>Buprenorphine</td>
<td>253</td>
</tr>
<tr>
<td>Rats</td>
<td>Systeic (rheumatic)</td>
<td>Pristane Injections</td>
<td>CatWalk</td>
<td>Print area, duration of stance phase, regularity index</td>
<td>Decrease in print area, decreased stance phase in 2-3 paws, decreased regularity index. Gait was partially restored at 4 weeks.</td>
<td>None</td>
<td>198</td>
</tr>
<tr>
<td>Rats</td>
<td>Knee</td>
<td>MIA</td>
<td>CatWalk</td>
<td>Max contact area, swing speed. Subtracted values from contralateral side</td>
<td>Significant right-left imbalances for max contact areas and swing speed.</td>
<td>Morphine</td>
<td>254</td>
</tr>
<tr>
<td>Species</td>
<td>Joint</td>
<td>Arthritis Model</td>
<td>Gait Method</td>
<td>Parameters Measured</td>
<td>Results</td>
<td>Therapies Tested</td>
<td>Citation</td>
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</tr>
<tr>
<td>Mice</td>
<td>Knee</td>
<td>DMM</td>
<td>CatWalk</td>
<td>Mean print intensity</td>
<td>No evidence of gait impairment</td>
<td>Morphine, Acetaminophen</td>
<td>255</td>
</tr>
<tr>
<td>Mice</td>
<td>Ankle</td>
<td>LPS</td>
<td>CatWalk</td>
<td>Paw pressure intensity, paw print area, and regularity index</td>
<td>Reduced paw pressure, but returned to normal after 4 days. Reduced print areas of all paws – all recovered except the injected limb. Paw pressure ratios and print area ratios decreased, and was most profound at 2 days.</td>
<td>Indomethacin</td>
<td>256</td>
</tr>
<tr>
<td>Rats</td>
<td>Paw</td>
<td>CFA</td>
<td>CatWalk</td>
<td>Ipsilateral load percentage (based on area and pressure intensity)</td>
<td>Load reduction on ipsilateral paw</td>
<td>None</td>
<td>257</td>
</tr>
<tr>
<td>Rats</td>
<td>Systemic (rheumatic)</td>
<td>Mycobacterium tuberculosis</td>
<td>CatWalk-like arena</td>
<td>Velocity, stride length, swing time, single stance time, double stance time</td>
<td>Decrease in velocity and stride length, increase in single and double stance time and swing time.</td>
<td>Muscimol, Bicuculline</td>
<td>258</td>
</tr>
<tr>
<td>Species</td>
<td>Joint</td>
<td>Arthritis Model</td>
<td>Gait Method</td>
<td>Parameters Measured</td>
<td>Results</td>
<td>Therapies Tested</td>
<td>Citation</td>
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</tr>
<tr>
<td>Rats</td>
<td>Knee or Ankle</td>
<td>CFA or Carra-geenan</td>
<td>CatWalk-like arena</td>
<td>Paw print intensity, guarding index, regularity index</td>
<td>CFA: Increased guarding index, reduced weight bearing and regularity index. Carrageenan: Increased guarding index, reduced weight bearing and regularity index</td>
<td>Naproxen, Diclofenac, Oxycodone, Ibuprofen</td>
<td>215</td>
</tr>
<tr>
<td>Rats</td>
<td>Paw</td>
<td>CFA</td>
<td>CatWalk-like arena</td>
<td>Velocity, hind paw stride length, stride time, swing time, single stance time, dual stance time, and duration of ground contact</td>
<td>Reduced velocity, stride length, single stance time, swing time, and ground contact. Increased dual stance time.</td>
<td>Buprenorphine</td>
<td>216</td>
</tr>
<tr>
<td>Rats</td>
<td>Knee</td>
<td>Carrageenan</td>
<td>DigiGait</td>
<td>Swing time, stance/swing ratio, braking time, stance time, % stance/stride, stride length, stride time, % swing/stride, % propulsion/stride, paw area, stance width</td>
<td>Injected limb: Decreased stance/swing ratio, stance, %stance/stride, stride length, paw area, stride. Increased %swing/stride, % propulsion/stride. Contralateral hind limb: Increased stance/swing ratio, % stance/stride. Decreased % swing/stride, % propulsion/stride. Ipsilateral forelimb: Decreased</td>
<td>None</td>
<td>225</td>
</tr>
<tr>
<td>Species</td>
<td>Joint</td>
<td>Arthritis Model</td>
<td>Gait Method</td>
<td>Parameters Measured</td>
<td>Results</td>
<td>Therapies Tested</td>
<td>Citation</td>
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</tr>
<tr>
<td>Mice</td>
<td>Knee</td>
<td>Mechanical</td>
<td>DigiGait</td>
<td>Stance phase, swing phase, stride time, stride length, paw area</td>
<td>Changes only seen in the contralateral limb. Increased stance and stride times, stride length, and paw area</td>
<td>None</td>
<td>259</td>
</tr>
<tr>
<td>Mice</td>
<td>Systemic (genetic)</td>
<td>STR/Ort mice</td>
<td>DigiGait</td>
<td>Stance phase, swing phase, stride time, stride length, paw area, brake and propel times, paw angle, symmetry index</td>
<td>Paw area is main parameter associated with OA.</td>
<td>None</td>
<td>227</td>
</tr>
<tr>
<td>Mice</td>
<td>Systemic (rheumat ic)</td>
<td>CIA</td>
<td>DigiGait</td>
<td>Paw area, paw angle, stride length, stride frequency, stride time, stance time, swing time, braking time, propulsion time</td>
<td>Increased stride frequency and paw area. Decreased stride length and stride time, paw angle, stance time, swing time, braking time, and propulsion time.</td>
<td>None</td>
<td>217</td>
</tr>
<tr>
<td>Species</td>
<td>Joint</td>
<td>Arthritis Model</td>
<td>Gait Method</td>
<td>Parameters Measured</td>
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<td>Therapies Tested</td>
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<td>--------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>Mice</td>
<td>Knee</td>
<td>Carra-geenan</td>
<td>DigiGait</td>
<td>Stride time, stance time, swing time, stride length, stride frequency</td>
<td>Increased swing time. No change in stance time, stride time, stride length, and stride frequency.</td>
<td>Buprenorphine</td>
<td>219</td>
</tr>
<tr>
<td>Mice</td>
<td>Knee</td>
<td>TFGβ1 and Running</td>
<td>Tread Scan</td>
<td>Stance time, Swing time, Braking time, propulsion time</td>
<td>No change in stride time, stride length, or stride frequency. Increased stance time, propulsion time, and swing time.</td>
<td>Hyaluronan</td>
<td>226</td>
</tr>
<tr>
<td>Rats</td>
<td></td>
<td>Systemic (rheumatic) Mycobacterium tuberculosis</td>
<td>Tread Scan</td>
<td>Stance time, swing time stride length, velocity</td>
<td>Reduced velocity, stride length, increased stance time and swing time</td>
<td>Indomethacin, Aurothiomalate, Chloroquine, D-penicillamine, Sodium Aurothiomalate, Methotrexate</td>
<td>218</td>
</tr>
<tr>
<td>Mice</td>
<td>Knee</td>
<td>Carra-geenan</td>
<td>Tread Scan</td>
<td>Stride time, stance time, swing time, stride length, stride frequency</td>
<td>No change in swing time. Increased stance time, stride time, and stride length. Reduced stride frequency.</td>
<td>Buprenorphine</td>
<td>219</td>
</tr>
<tr>
<td>Rats</td>
<td>Knee</td>
<td>Overuse Injury</td>
<td>Ink Prints</td>
<td>Stride length and step angles</td>
<td>No difference in stride length, decreased paw angles</td>
<td>None</td>
<td>260</td>
</tr>
<tr>
<td>Species</td>
<td>Joint</td>
<td>Arthritis Model</td>
<td>Gait Method</td>
<td>Parameters Measured</td>
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<td>Rats</td>
<td>Knee</td>
<td>CFA</td>
<td>Ink Prints</td>
<td>Limb rotation, stride length, stance width</td>
<td>Increased foot rotation and stance width, reduced stride length</td>
<td>NBQX</td>
<td>261</td>
</tr>
<tr>
<td>Rats</td>
<td>Knee</td>
<td>CIA</td>
<td>Ink Prints</td>
<td>Step lengths, angles, and walking speed</td>
<td>Asymmetric gait, decreased step lengths, and increased paw angles</td>
<td>Enbrel (anti-TNF)</td>
<td>202</td>
</tr>
<tr>
<td>Mice</td>
<td>Knee</td>
<td>ACLT and PCL Transection</td>
<td>Ink Prints</td>
<td>Stride length, base of support, paw print area</td>
<td>No change in stride or base of support, smaller foot print after transection</td>
<td>None</td>
<td>224</td>
</tr>
<tr>
<td>Mice</td>
<td>Knee</td>
<td>Systemic (genetic) GDF5 deficient mice</td>
<td>Ink Prints</td>
<td>Stride length, base of support</td>
<td>Reduced stride length, no change in base of support</td>
<td>None</td>
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</tr>
<tr>
<td>Rats</td>
<td>Knee</td>
<td>MMT</td>
<td>Custom Gait Arena</td>
<td>Stride length, step width, duty factor, gait symmetry</td>
<td>Asymmetric gait after MMT, imbalanced, different stance time, no change in stride length, step width, and stride frequency</td>
<td>None</td>
<td>188</td>
</tr>
<tr>
<td>Mice</td>
<td>Systemic (genetic) Type IX collagen inactivation</td>
<td>Custom Gait Arena</td>
<td>Velocity, duty factor, stride length, step width, stride frequency, and symmetry</td>
<td>Reduced velocity, increased duty factor, shorter stride lengths, and wider step widths</td>
<td>None</td>
<td>209</td>
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<tr>
<td>Species</td>
<td>Joint</td>
<td>Arthritis Model</td>
<td>Gait Method</td>
<td>Parameters Measured</td>
<td>Results</td>
<td>Therapies Tested</td>
<td>Citation</td>
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<td>---------</td>
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<tr>
<td>Rats</td>
<td>Knee</td>
<td>IL-1β over-expression</td>
<td>Custom Gait Arena</td>
<td>Velocity, stride length/stride frequency, step width, toe-out angle, duty factor, and gait symmetry</td>
<td>Reduced time spent on affected limb and gait symmetry greater than 0.5. Velocity increased, stride lengths increased, and toe-out angles trended up with time.</td>
<td>IL1Ra</td>
<td>197</td>
</tr>
<tr>
<td>Rats</td>
<td>Knee</td>
<td>MMT</td>
<td>Custom Gait Arena</td>
<td>Velocity, stance time, swing time, stride time, stride length, and step width. Calculated spatial symmetry, temporal symmetry, duty factor, stance time imbalance, percentage single-limb support.</td>
<td>Asymmetric gait at 2 and 6 weeks post-surgery. Narrow step widths at 2, 4, and 6 weeks. Reduced stride length residuals and 4 and 6 weeks. Temporal asymmetry at 1 and 4 weeks. Stance time imbalance at 1 and 6 weeks.</td>
<td>None</td>
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<td>Rats</td>
<td>Knee</td>
<td>Carrageenan</td>
<td>Running Wheel</td>
<td>Swing time, swing time ratio</td>
<td>Decreased swing time ratio and swing time</td>
<td>Indomethacin</td>
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<tr>
<td>Mice</td>
<td>Systemic (genetic) Type IX collagen inactivation</td>
<td>Running Wheel, Treadmill, and</td>
<td>Contact time, stride time, duty factor, stride frequency,</td>
<td>Slower wheel running speed, no difference in duty factor, stride frequency. Overground: No difference in</td>
<td>None</td>
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Table 6-3. Continued

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<tr>
<th>Species</th>
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<th>Gait Method</th>
<th>Parameters Measured</th>
<th>Results</th>
<th>Therapies Tested</th>
<th>Citation</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Custom Gait Arena</td>
<td>instantaneous speed</td>
<td>duty factor, stride frequency. Wheel had faster running speeds than over ground, lower duty factor. No difference in stride frequency.</td>
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</table>

MIA: Monoiodoacetate Injection; CFA: Complete Freud’s Adjuvant Injection; CIA: Collagen Induced Arthritis (via antigen); LPS: Lipopolysaccharide Injections; ACLT: Anterior Cruciate Ligament Transection; MMT: Medial Meniscus Transection (central tear); DMM: Destabilized Medial Meniscus (at anterior horn
<table>
<thead>
<tr>
<th>Species</th>
<th>Animal Model</th>
<th>Gait Method</th>
<th>Parameters Measured</th>
<th>Results</th>
<th>Therapies Tested</th>
<th>Citation</th>
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<tr>
<td>Rats</td>
<td>MMT</td>
<td>AMTI Force Plates</td>
<td>Force in x, y, and z. Impulses</td>
<td>Peak vertical force and impulse decreased, propulsive forces and impulse decreased. No change in braking and mediolateral forces.</td>
<td>None</td>
<td>188</td>
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<tr>
<td>Rats</td>
<td>Carra-geenan</td>
<td>Dana Load Cells</td>
<td>Vertical Force</td>
<td>Reduced loads after injection. Returned to baseline after 5 days</td>
<td>Morphine</td>
<td>264</td>
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<tr>
<td>Rats</td>
<td>Varus Loading and Compres-sive Overload</td>
<td>JR3 Load Cell</td>
<td>Force in x, y, and z</td>
<td>Attachment of device: 30% reduced vertical force, reduced A/P force, and M/L shifted. Increased contact time. Overloading: Experimental vs contralateral legs different for all measures except contact time.</td>
<td>None</td>
<td>265</td>
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<tr>
<td>Rats</td>
<td>MIA</td>
<td>Tekscan Walkway</td>
<td>Weight bearing</td>
<td>Reduced weight bearing after MIA.</td>
<td>Naproxen Sodium, Morphine</td>
<td>266</td>
</tr>
<tr>
<td>Rats</td>
<td>MIA</td>
<td>Tekscan Walkway</td>
<td>Weight bearing</td>
<td>Reduced weight bearing after MIA. Compensated by increased weight bearing in contralateral hind limb.</td>
<td>Dexamethasone, Celecoxib, Duloxetine, Naproxen, Morphine, Pregabalin</td>
<td>267</td>
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<tr>
<td>Rats</td>
<td>ACLT</td>
<td>Tekscan Walkway</td>
<td>Paw pressure, impulse</td>
<td>Increased left to right hind limb average maximum force ratio</td>
<td>Human Synoviocyte Lubricin</td>
<td>201</td>
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<tr>
<td>Rats</td>
<td>MMT</td>
<td>Dynamic</td>
<td>Percentage body</td>
<td>MMT animals shifted weight to</td>
<td>None</td>
<td>268</td>
</tr>
<tr>
<td>Species</td>
<td>Animal Model</td>
<td>Gait Method</td>
<td>Parameters Measured</td>
<td>Results</td>
<td>Therapies Tested</td>
<td>Citation</td>
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<tr>
<td>Mice</td>
<td>CFA</td>
<td>Dynamic Weight Bearing (Bioseb)</td>
<td>Percentage body weight on hind paws</td>
<td>Significant changes in load distribution were seen as a function of injection concentration.</td>
<td>Indomethacin, Dexamethasone, Morphine Fluorocitric Acid, Minocycline</td>
<td>231</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weight Bearing (Bioseb)</td>
<td>weight and surface area of each paw</td>
<td>forepaws sooner and the forepaw surface area increased sooner. MMT also increased more weight of the contralateral hind limb.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MIA: Monoiodoacetate Injection; CFA: Complete Freud's Adjuvant Injection; ACLT: Anterior Cruciate Ligament Transection; MMT: Medial Meniscus Transection (central tear)
Collecting and analyzing rodent gait must be done with precision and care. Since rodents are orders of magnitude smaller than humans, a small misplacement of the camera or incorrect lighting can introduce significant error or invalidate an experiment. The Orthopaedic Biomedical Engineering Lab at the University of Florida developed a custom rodent gait analysis system, which has progressed over many years and iterations.

In overview, the gait analysis system includes a bottom structure which holds a mirror at 45°, a secondary clear instrumented floor above the mirror, and an animal enclosure on top of the secondary instrumented floor, as shown in Figure 7-1 (next page). When an animal is placed in the enclosure, a high-speed video camera captures the lateral and ventral views of the animal, while the instrumented floor collects ground reaction forces when the animal strikes a force panel. This system has been named the Experimental Dynamic Gait Arena for Rodents (EDGAR). Data from EDGAR is processed through a rodent specific code, termed Automated Gait Analysis Through Hues and Areas (AGATHA), which isolates the animal from the video and measures spatiotemporal gait parameters from foot strikes and paw placements. Several additional codes were created to isolate force curves from force plate recordings.
Figure 7-1. A model of EDGAR is shown with major components. Animals are placed into the animal enclosure and are allowed to freely explore. A high-speed video camera (not shown) records the frontal view of the area, while the force plate instrumented floor records force data.

With EDGAR, lighting of the gait arena and animal is critical for post-processing videos with the AGATHA algorithm. Ideal lighting should be even across the arena, with no bright or dark spots. Lighting must also provide maximum contrast to differentiate the animal from the arena background, and the animal’s paws from the fur. Additionally, lighting must be consistent throughout an experiment such that post-processing is easier. Originally, 1000W work lights were used to light the gait arena; however, these lights had numerous problems. Work lights can provide even lighting across the arena, but this requires at least 6 lights on 3 different stands and at least an hour of time to angle all lights the proper way. Also, work light stands take up a lot of space, which makes moving animals into and out of the testing arena very difficult. While work lights provide warm light and are sufficient for creating contrast between the animal and the background, the high-speed camera picks up the natural electronic flicker from these
lights at high frame rates. Additionally, the work lights can sag and change position over time, making it difficult to keep consistent lighting between days. However, the single biggest problem with work lights is the extreme heat, causing the room to be very hot and uncomfortable for both the scientist and the animals.

Thus, EDGAR was redesigned to incorporate light emitting diode (LED) bar lights, which produce minimal heat. However, traditional LEDs also flicker due to alternating current power supplies, which will appear on a high frame rate video. Thus, our LED light bars are connected to a direct current (DC) power supply to reduce or eliminate flicker, much like a car’s headlights being connected to the car battery. Lights were also directly attached to the arena, keeping the lights in position, and eliminating the stands from the room.

Additional improvements to EDGAR were made in regard to force plate mounting. Previously, the force plates were secured to metal bars with acrylic; however, the acrylic was not accurately machined, limiting the rigidity of the force plate attachment. Therefore, each force plate attachment was acting as a cantilever. To correct this, a new design was created to sandwich the force plates with two panels of acrylic. The bottom panel is secured to the metal bars, while the top panel is only secured to the force plates, which the animal will step on. Finally, the layout of the force panels was changed to have two pairs of consecutive force panels, which increased the number of steps collected per gait trial.

The final area of improvement was made in the post-processing code, AGATHA. Prior iterations of the code were strongly based on the individual using it at the time. Thus, the code was not easily transferable to other studies. Additionally, the code
required high frame rate videos to be run multiple times to get the correct filtering. With the improved GAITOR Suite (Gait Analysis Instrumentation and Technology Optimized for Rodents, Figure 7-2, next page), a filtering subroutine is done up front, the AGATHA subroutine algorithm is performed, and then a gait parameter calculator is done on the back end. The filtering subroutine on the front end allows lighting conditions to be altered for each day of recording. Once the filters are set, the AGATHA algorithm will automatically run gait videos and output all data required to calculate the spatiotemporal gait parameters. Following the AGATHA algorithm, all files may be quickly run through the back end calculator code to compile results. Additionally, a back end editor code was written, allowing the user to pull data from the AGATHA algorithm, edit the trial to consider or ignore specific regions of the trial, then re-analyze the trial. This editing code does not require the entire video to be processed again, unlike the original AGATHA algorithm. Additionally, a dynamic code to analyze ground reaction forces was heavily modified to work in combination with the GAITOR Suite, and is now another modular component for the system. The dynamic code has improved user interfaces and options for collecting fore and hind limb force curves.

Finally, GAITOR Suite is now openly available at www.GAITOR.org. Here, other laboratories can get instructions to build EDGAR, get information on hardware, and download the complete code suite needed for processing gait data. Two external labs are already running experiments using the GAITOR Suite platform, both of which are neurological models. Thus, GAITOR Suite is becoming a tool for varying fields to quantify preclinical behavior and mechanics in rodents.
Figure 7-2. An outline for the GAITOR Suite of code for rodent gait analysis is shown. The code is modular and easily customizable depending on user needs. Figure by Brittany Jacobs.
CHAPTER 8
QUADRUPEDAL GAIT COMPENSATIONS IN A LOW DOSE MONOIOACETATE MODEL OF OSTEOARTHRITIS

Motivation for Quantifying Rodent Gait Compensations after a Low Dose of MIA

Osteoarthritis is the leading cause of disability in America, causing pain and disability in 23% of adults\textsuperscript{269,270}. Currently, clinical osteoarthritis management focuses on reducing pain and restoring joint function, as measured by patient pain scoring and passive range of motion\textsuperscript{271–273}. However, active mechanics of the limb may further characterize compensatory behavior and/or instability, which may also relate to pain. For example, injury and degeneration of the knee can cause multiple changes in the joint, including altered load distribution and would healing responses. Moreover, a joint injury may cause dysesthesia or altered proprioception in the joint, resulting in altered gait.

Gait analysis is a common method to quantify changes in orthopaedic diseases. While gait analysis is traditionally performed in humans, preclinical rodent gait analysis is becoming popular. Rodent gait analysis is an operant and largely stress-free method for recording natural behavior in preclinical studies. Additionally, gait analysis provides robust, quantitative results for characterization of musculoskeletal models.

Custom gait recording methods for rodents have collected robust spatiotemporal gait parameters via high speed cameras while simultaneously recording ground reaction forces\textsuperscript{188–190}. Prior work introduced a custom rodent gait system, termed EDGAR (Experimental Dynamic Gait Arena for Rodents, described in Chapter 7)\textsuperscript{189}. EDGAR was used to evaluate gait in rats with either medial meniscus transection (surgical model) or intra-articular injection of monooiodoacetate (3mg in 25µL saline, chemical
model). Results from this study showed the surgical model resulted in a shuffle-step, while the chemical model resulted in antalgic gait\textsuperscript{189}. MIA is a glycolysis inhibitor which causes widespread joint damage, and 3mg of MIA is on the high end of what is traditionally used in joint degeneration models\textsuperscript{168,251,252,254,266,267,274,275}. Thus, the varying gait compensations observed in the 3mg MIA model could be caused by the higher chemical dose or by the model causing significantly more joint damage than observed at lower doses. Thus, it is unknown whether the antalgic gait caused by MIA is associated with severity of degeneration or the general and broad destruction of the joint associated with glycolysis inhibition.

Since the severity of damage observed in the MIA model can be titered with dose, this chapter aims to evaluate gait changes resulting from 1mg of MIA. Again, EDGAR will be used to evaluate rodent gait compensations, but since gait changes may be mild in a lower dose of MIA, gait analysis in the GAITOR Suite (described in Chapter 7) was adapted to uniquely quantify both fore and hind limb gait parameters. Our data demonstrate gait abnormalities change with MIA dose, where compensations subsequent to 1mg of MIA were relatively minor and tended to follow the shuffle-step compensations observed in prior surgical models.

**Methods for Inducing Joint Degeneration and Subsequent Gait Testing and Analysis**

**Experimental Design for Low Dose MIA**

Animal use was approved by the University of Florida’s Institutional Animal Care and Use Committee. Male Lewis rats were used (n = 42, Charles Rivers Laboratories, Wilmington, MA, USA). Animals were divided into the following three groups:
monoiodoacetate (MIA) injection (n = 6 per time point, 250-400g), saline injection (n = 6 per time point), or naïve (n = 6 total, 250-400g). Six animals from each group underwent gait testing at 1, 2, and 4 weeks post-injection. After gait testing at each time point, the same 6 MIA and 6 saline animals that were gait tested were euthanized, while the 6 naïve animals (300-450g) were tested at each time point and euthanized at the end. Following euthanasia, knee joints were dissected and processed for histology. Experimental design is summarized in Figure 8-1.

![Diagram of experimental design](image)

Figure 8-1. Experimental design for evaluating rodent gait after intra-articular (IA) injection with monoiodoacetate or saline. Six animals from each group were gait tested at 1, 2, and 4 weeks post-injection. Animals tested that week were euthanized. Additionally, a continuous group of six naïve animals were tested each week, but were not euthanized until the study concluded.
MIA Intra-articular Injection Procedure

Animals were placed in an induction chamber with 4% isoflurane in oxygen. Anesthesia was maintained via mask inhalation of 3% isoflurane during each procedure. The right hind limb was shaved and aseptically prepared using povidone-iodine and alcohol in triplicate, with a final application of povidone-iodine left on the skin. Animals were then given an intra-articular injection of either MIA or sterile saline, as described below.

For MIA and saline injections, a 29 gauge insulin syringe was inserted through the superior portion of the patellar ligament and followed the femoral groove behind the patella until the needle was in the joint space. After initial insertion, the needle tip was rotated obliquely with the bevel directed toward the joint space. Then, for MIA injections, 1 mg of MIA suspended in 25 µL of sterile saline was delivered (n = 18 total). Similarly, for saline injections, 25 µL of sterile saline alone was delivered (n = 18 total). Animals recovered in a warming box until weight bearing on all limbs. Animals were given buprenorphine for 48 hours post-surgery.

Gait Analysis in Rats

The Experimental Dynamic Gait Arena for Rodents (EDGAR, described in Chapter 7) is designed to simultaneously collect spatiotemporal and dynamic ground reaction gait data from freely walking rodents (also shown in Figure 8-2, page 122). Specifications for EDGAR are openly provided at www.GAITOR.org. Briefly, EDGAR requires an unobstructed view of the lateral and ventral planes of the animal, achieved by placing a mirror at 45° below the arena floor. To allow simultaneous dynamic data collection, a secondary floor, constructed from a series of transparent instrumented
panels and non-instrumented floor sections, is placed above the main floor. Instrumented panels are placed between non-instrumented sections, which are then supported by a rigid frame and damping material. Each instrumented panel measured 6.35 cm by 24.13 cm, with a 3-component force-link (Type 9317B Kistler, Winterthur, Switzerland) mounted on each corner of the panel.

To calibrate EDGAR, known weights were placed on each instrumented panel (20, 50, 100, 200, and 500g) in the vertical (Z) direction. Signal from the charge amps (Type 5010 Dual Mode Amplifier, Kistler, Winterthur, Switzerland) was collected using custom LabVIEW code. Calibration data was then used to verify linear behavior of the force plates and determine charge-gram conversion factor for each panel on each testing day.

The animal enclosure placed on top of the secondary floor measured 14 cm wide by 150.5 cm long by 25.5 cm tall with a hinged lid. Spatiotemporal gait videos were captured with a high-speed camera at 500 fps (M3 Redlake, San Diego, CA, USA). Ground reaction forces were collected through a custom LabVIEW code, and paw strikes on the instruments panels were verified with video.
Figure 8-2. Gait collection using EDGAR. A) The side view shows an animal walking down the arena, with a mirror oriented 45 degrees below the clear acrylic floor. B) Two force panels were created to collect vertical ground reaction forces as the animal crosses the arena, as shown in the frontal view. Photo courtesy of author.

Animals underwent gait testing at 1, 2, and 4 weeks post-injection. Animals were tested in random order to minimize the effect due to time of day. Gait testing was conducted as follows: each animal was placed in the arena and allowed to explore until either 10 foot strikes were recorded on the force panel for right and left hind paws, or until the animal reached 20 minutes per session.

Spatiotemporal gait data were processed using our lab’s gait analysis software GAITOR Suite, previously described in Chapter 7. Briefly, GAITORSuite may be used
to determine both spatiotemporal and dynamic gait parameters, as described in our methodological reviews\textsuperscript{187,276}. Both hind and fore paws were examined in spatiotemporal and dynamic data.

**MIA versus Saline Histology**

At each time point, animals were euthanized and hind limbs were collected for histology. Collected joints were fixed in 10% neutral buffered formalin for 48 hours, decalcified in Cal-Ex Decalcifier (Fisher Scientific, Pittsburg, PA, USA) for 3 weeks, and then paraffin embedded using vacuum infiltration (Tissue-Tek VIP 6, Sakura Finetek, Torrance, CA, USA). Frontal sections were taken at 10 µm starting after the anterior horn of the medial meniscus through to the posterior horn. Sections representing the loading region were stained with toluidine blue.

**MIA Gait Data Post-Processing and Statistics**

One naïve animal was removed from the data set as an outlier, which was confirmed through notations that this animal refused to voluntarily walk in the gait arena (leaving n = 5 naïve animals at each time point). To improve the naïve data set, all right and left data were combined for the fore and hind limbs, since asymmetries are not expected in naïve animals. Since naïve animals were of a different age and size at each time points, a control line was calculated that related velocity and body weight to various gait parameters, when indicated (all gait parameters except temporal symmetry, spatial symmetry, and duty factor imbalance). Using this process, the expected result for a naïve animal at a given body weight and velocity can be calculated as follows:

\[
\text{Expected Result} = \beta_0 + \beta_1 \times \text{Velocity} + \beta_2 \times \text{BW} + \beta_3 \times \text{Velocity} \times \text{BW} \quad (8-1)
\]
This residual calculation process is further described in Figure 8-3. After spatiotemporal and dynamic data were in final form, median gait parameters were calculated for each animal to ensure no animal was weighted more than another due to differences in trial numbers. From animal medians, a Kruskal-Wallis test was used to compare multiple independent samples between saline and MIA injected animals ($\alpha = 0.05$). To compare saline and MIA injected animals to expected values, a sign test was used ($\alpha = 0.05$).

![Diagram of residual calculation process]

Figure 8-3. Covariates for the data are determined by plotting the data against each predictor. For this study, both velocity and body weight were used as covariates to residualize experimental data.
Results of Low Dose MIA Injections

MIA Histology Results

Representative histological images at each time point are shown in Figure 8-4. MIA knees showed more regions of cell death in the tibial cartilage, where cells were no longer present. This continued into week 4, where delamination at the bone-cartilage interface began to occur. Saline animal histology was mostly normal; however some evidence of cell cloning was observed.

Figure 8-4. Toluidine blue staining is shown for saline and MIA animals at each testing week (10 µm sections). Naïve animals were not euthanized at each time point. MIA histology shows areas of cell death at all weeks (arrows), and the beginning of cartilage delamination at week 4 (*). Saline animals unexpectedly showed cellular cloning (C) and some evidence of cell death (arrows) at week 4. Photos courtesy of author.
MIA Spatiotemporal Results

Average group velocities were 39.7±7.2 cm/s for saline animals and 38.2±6.2 cm/s for MIA animals (p = 0.1968). Temporally, saline animals had significantly lower left fore limb duty factors at week 1, as indicated by all points being below the control line in Figure 8-5A (next page). Generally, fore limb temporal symmetry was higher in both groups at all weeks (all median bars above the 0.5 line in Figure 8-5B, not statistically significant). For hind limbs, MIA animals showed a significantly higher hind duty factor imbalance at week 2, as shown by the negative sloping lines connecting left and right means in Figure 8-5C (next page). At week 4, both saline and MIA animals had an increased duty factor of the left hind limb compared to the control line (all points above control line, p = 0.0313). No temporal differences between saline and MIA animals at a given week.
Figure 8-5. Temporal results are shown as scatterplots, where the left and right duty factors are shown paired for the same animal, as shown by the connecting lines on duty factor plots. These lines represent the duty factor imbalance. Solid lines for each scatterplot set represent the median for each group and foot. * indicates a significant difference from the control line, while ∧ represents a significant imbalance between right and left (sign test, p < 0.05).
Figure 8-6. Spatial results are shown as representative figures on the left and scatterplots on the right. In representative spatial figures, naïve data is shown for reference (black). Average naïve data was determined by combining right and left data from all weeks. In scatterplots, the naïve data is represented by the solid horizontal lines showing the expected value. Sign tests were used to compare groups to these lines (* indicates p < 0.05). Solid lines for each scatter plot set represent the median for each group and foot.
Spatially, both MIA and saline animals had decreasing stride lengths over time, where saline animals had significantly longer stride lengths at week 1, and MIA animals had significantly shorter stride lengths at week 4 (relative to control line in Figure 8-6B on previous page, \( p = 0.0313 \)). No significant differences were seen in fore limb step widths, but hind limb step widths were wider at week 2 for both MIA and saline animals (relative to control line in Figure 8-6C). MIA animals maintained a wider hind limb step width (still higher at week 4), while saline animals appeared to reach an expected step width at week 4 (median near control line in Figure 8-6D).

**MIA Dynamic Results**

As shown in Figure 8-7 (next page), no significant differences were seen in fore limb loading or peak vertical impulse at any week. Fore hind limbs, MIA animals showed a decrease in right hind peak vertical force at week 1, as shown by all points being below the control line in Figure 8-7C (\( p = 0.0313 \)). MIA animals at week 1 were also significantly imbalanced, as shown by the negative slope of lines connecting left and right peak vertical forces. MIA animals continued to be imbalanced at week 2, but overall hind loading was shifted up such that hind left peak vertical forces were significantly above the control line. MIA animals returned to a balanced loading of the hind limbs by week 4, while saline animals began to be imbalanced at week 4. No differences were seen in peak vertical impulse of the hind limbs.
Figure 8-7. Dynamic loading results are shown as scatterplots, where the left and right peak vertical forces (left) and impulses (right) are shown paired for the same animal, as shown by the connecting lines. These lines represent the peak vertical force and impulse imbalances. Solid lines for each scatterplot set represent the median for each group and foot. * indicates a significant difference from the control line, while ∧ represents a significant imbalance between right and left (sign test, p < 0.05).
For timing of dynamic events, both MIA and saline animals took longer to reach peak vertical force on the fore limbs at week 1, as shown by points being above the control line in Figure 8-8A (next page, \( p = 0.0313 \)). For hind limbs, saline animals took less time to reach peak vertical force of the left hind limb compared to the control line and also compared to the right limb loading, shown as an imbalance in of Figure 8-8B. Saline animals also had significantly shorter times to load the left hind limb at week 4. MIA animals had balanced loading times at each week. Looking at the time difference between fore and hind peak vertical forces, both MIA and saline had significantly less time between peaks at week 2, indicating more overlap in fore to hind limb transition. These timings mostly returned to normal by week 2, with only saline animals having more time between right fore to hind limb transitions. No differences were found between saline and MIA animals at a given week.
Figure 8-8. Dynamic timing results are shown as scatterplots, where the left and right time to peak vertical force and the time difference between fore and hind peak vertical forces are shown paired for the same animal, as shown by the connecting lines. These lines represent the timing imbalances. Solid lines for each scatter plot set represent the median for each group and foot. * indicates a significant difference from the control line, while ∧ represents a significant imbalance between right and left (sign test, p < 0.05).
Discussion for Low Dose MIA

Gait compensations after injection of 1mg of MIA tended to follow the gait profile of a surgical medial meniscus transection (MMT). At week 1, MIA animals tended to exhibit an antalgic gait pattern, as indicated by imbalanced duty factors where animals spent less time on their right (injected) limb and lower peak vertical forces on the right (injected) limb. While the spatial pattern was symmetric in MIA animals at weeks 1 and 2, the hind peak vertical force was imbalanced, offloading the right (injected) limb. Imbalanced limb loading with a symmetric gait pattern was previously observed in early stages of the MMT model\textsuperscript{189}. Like our previous reports, these data indicate the importance of measuring dynamic data in combination with spatiotemporal data, as compensatory gait patterns may not be captured by only spatial, temporal, or dynamic characterizations alone.

Over time, MIA animals transitioned to shuffle-stepping, as indicated by a decrease in stride length, balanced but raised hind limb duty factors, and a wider hind step widths. The combination of these changes indicate animals are taking shorter steps, spending more time on each foot as they walk, and widen their base of support for stability. Again, shuffle-stepping was previously seen in the MMT model\textsuperscript{189}. Prior literature has attributed shuffle-stepping to a compensation which reduces limb loading\textsuperscript{277,278}. Thus, we expected the hind limb peak vertical forces to be balanced between right and left feet, but lower compared to age and weight-matched control animals. While we observed balanced hind limb forces at week 4, these forces were not significantly lower than the expected control line. Here, force curves at week 1 were all lowered compared to naïve (Figure 8-9, page 135), but these decreases in force are less obvious by week 4. It is important to realize that force curves in Figure 8-9 are not
velocity corrected, and are intended as a visual representation of the measured gait pattern. However, velocity and body weight corrected data in Figure 8-7 still indicates minimal changes in peak vertical force despite spatiotemporal patterns indicative of a shuffle-step.

While the aim of this chapter was to evaluate a low dose of MIA to compare with high doses of MIA and surgical models, joint damage in this study was milder than intended (Figure 8-4). However, even with mild joint damage present in histology, we detected evidence of a gait abnormality relative to age and weight matched controls. Interestingly, similar gait changes were also seen in the saline injected group, indicating that saline injections in rodents may not be as harmless as typically thought. This may be explained by the injection procedure itself causing inflammation and irritation of the joint.

To our knowledge, this chapter provides the first detailed 4 limb analysis of rodent gait, including spatiotemporal and dynamic data from the same gait trial. This chapter presents all gait measures for each limb, and we were able to create a cohesive graph showing the relationship between limb loading and timing of the gait pattern in Figure 8-9 (next page). While these methods provide additional detail on the rodent gait pattern, the question remains as to whether analyzing the fore limbs adds meaningful data to preclinical models. In this study, fore limb data showed no significant changes in spatial parameters. However, this was largely expected as spatial patterns in the fore limbs are heavily linked to the hind limbs, especially since stride lengths must be equal for all 4 limbs. Dynamically, the fore limb peak vertical force and impulse were largely unaffected, but significant changes were seen in both saline and MIA animals in the
timing of the peak vertical force of the fore limbs (Figure 8-8). It appears that at 1 week post-injection, animals tend to load both fore limbs more slowly. This fore limb time to peak force remained higher in MIA animals at all weeks, while saline injected animals returned to a normal loading time by week 4. This indicates fore limbs are loading more slowly in MIA animals, which could be associated with lower propulsive forces being generated in the hind limbs. This, again, would be associated with a shuffle-step in MIA animals; however, it is important to note the EDGAR set-up used in this work was unable to capture braking and propulsive forces. Thus, while the spatiotemporal and vertical forces in this chapter indicate lower propulsive forces in the hind limbs of MIA animals, this finding should be confirmed with direct measurement of braking and propulsive forces in future studies.

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Dynamic graphs were created by averaging all curves for each group on each respective foot, with the exception of naïve graphs. Naïve animals were averages across left and right limbs and across weeks to provide a weight matched control. Temporal data are shown as Hildebrand plots, where solid bars represent the duration of foot contact, with white bars indicating standard deviations of stance time.

Figure 8-9. Dynamic and temporal data are shown as percentage of the gait cycle. Dynamic graphs were created by averaging all curves for each group on each respective foot, with the exception of naïve graphs. Naïve animals were averages across left and right limbs and across weeks to provide a weight matched control. Temporal data are shown as Hildebrand plots, where solid bars represent the duration of foot contact, with white bars indicating standard deviations of stance time.
Furthermore, the time difference between fore and hind peaks was lowered in both saline and MIA animals at week 1, indicating more overlap in fore and hind limb loading. This may be the strongest evidence from this study that the fore limbs play a role in compensating for the hind limbs. However, our results indicate this compensation may only occur at the earliest time point, and may be associated with the injection procedure. Future studies should further explore these timing events at time points beyond 4 weeks for confirmation that the change is associated with the procedure and not subsequent degeneration.

Temporally, there was a tendency for fore limb duty factors to be increased in 1mg MIA at 4 weeks, though this change was non-significant. This relates to increased duty factors on the hind limbs, indicating both limb pairs are shuffle-stepping. This result is logical from a gait coordination standpoint, because otherwise, the synchronization of the hind limbs and fore limbs would be changing over multiple gait cycles. However, the degeneration caused by 1mg of MIA was very mild; thus, it is not clear whether the fore limbs would compensate for the hind limbs for more severe injuries.

Interestingly, hind limb time to peak vertical force was imbalanced at 1 week in saline animals, indicating the left hind limb is loading faster than the right (injected) limb (Figure 8-8). This change resolved by week 2, and it is not apparent why this occurs in saline animals but does not occur in MIA animals. Saline was used as a vehicle to deliver MIA; therefore, significant changes in saline only were not expected. Again, this may indicate that saline injections into the knee may not be as harmless typically thought.
Conclusions for Low Dose MIA

In summary, this study examined the fore and hind limb spatiotemporal and dynamic gait parameters of rats given intra-articular injections of either saline or 1mg of MIA. Our data indicate gait compensations are dependent on MIA dose, where animals injected with 1mg of MIA developed shuffle-step compensations, similar to the previously reported compensation in a surgical model. Additionally, this work provided detailed spatiotemporal and dynamic gait parameters for all 4 limbs of a walking rodent, which suggested fore limb compensations for the hind limbs.
CHAPTER 9
EVALUATION OF MALE AND FEMALE GAIT COMPENSATIONS IN MICE AFTER DESTABILIZATION OF THE MEDIAL MENISCUS

Introduction to Mouse Osteoarthritis Models and Outcomes

In humans, osteoarthritis pain levels do not always predict the severity of joint degeneration, as patients with severe pain can have relatively little cartilage degeneration, while patients with little pain may have significant cartilage degeneration\textsuperscript{157}. Additionally, many biological, post-mortem, and invasive measures cannot ethically be obtained from human patients. Preclinical animal models allow the progression of osteoarthritis to be examined at multiple time points and offer the opportunity to measure biological changes that cannot be ethically measured in humans. However, it remains difficult to simultaneously study disease symptomology and pathology in animals.

Gait analysis is emerging as a robust behavioral test that allows an animal to move naturally while providing quantitative data on the animal’s selected gait pattern. While gait testing can be time consuming, the results are quantitative, operant measures of rodent behavior, which can be used to discern how animals compensate for osteoarthritis\textsuperscript{187}. Our lab has developed a quantitative method for measuring functional behavioral responses in rodents via an Experimental Dynamic Gait Arena for Rodents (EDGAR, described in Chapter 7). To date, EDGAR has been successful in discerning measurable differences between naïve rats and rats with osteoarthritis\textsuperscript{189}. However, EDGAR has not yet been used for gait analysis in mice. Since mice are a more common preclinical animal model, gait analysis would be extremely useful to quantify behavioral changes in these osteoarthritis models of osteoarthritis. Thus, the
goal of this chapter is to evaluate the applicability of EDGAR for a common mouse model of osteoarthritis.

DMM Experimental Design and Methods

DMM Surgery

Destabilization of medial meniscus (DMM) is a commonly used surgical model for inducing osteoarthritis in mice. The transection of the medial meniscotibial ligament destabilizes the meniscus, creating instability in the joint. This instability initiates post-traumatic osteoarthritis remodeling in the joint, which ultimately develops into joint degeneration that is similar to clinical osteoarthritis\(^{279}\). In this study, a total of 20 C57/Bl6 mice of mixed gender were used to study the effects of DMM surgery on rodent gait.

Animals were obtained from Charles River Laboratories (Wilmington, MA, USA) and acclimated for one week in the University of Florida housing facilities. Ten mice (five male and five female) received a destabilized medial meniscus (DMM) surgery and ten mice (five male and five female) received a sham surgery, as described below. All surgeries were performed on the right hind leg on the same day. Surgeries were performed by the same person who remained blinded to the operation until the divergent moment in the surgery. Animals were anesthetized in a 4% isoflurane sleep box, prepared for aseptic surgery, and transferred to a sterile field with anesthesia maintained by mask inhalation of 2% isoflurane\(^{190}\).

For both DMM and sham surgeries, a medial para-patellar ligament incision was made on the right hind leg. The fat pad was bluntly dissected to reveal the medial meniscus and the medial meniscotibial ligament. At this point, sham animals were
closed and post-surgery procedures began. For the group receiving DMM surgery, the meniscotibial ligament was transected and incisions were closed and post-surgery procedures began\textsuperscript{280}. Incision sites were closed with 5-0 vicryl sutures (Ethicon, Somerville, NJ, USA) which were removed before behavioral testing.

Dynamic force data and spatiotemporal gait data were collected for all mice at 2, 4, 6, 9, and 12 weeks post-surgery, as described in the following section. After week 12, animals were euthanized, and right hind knee joints were collected for histological processing. Experimental design is summarized in Figure 9-1.

All methods were approved by the University of Florida Institutional Animal Care and Use Committee in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care recommendations on animal research.
Dynamic Force and Spatiotemporal Data Collection

Before testing, animals were acclimated to the gait arena by independently exploring the arena for 5 minutes every day for 3 days before data collection began. The room was set up as if testing were occurring to simulate actual testing conditions as much as possible. To collect data, animals were placed in the gait arena and allowed to freely explore without incentives or other external stimuli. High speed videos were recorded (M3, 500 frames/s; RedLake, San Diego, CA, USA) as the animal crossed the arena and walked over the force panels. Videos with non-repeatable gait patterns were excluded. Simultaneously, forces were collected from the Kistler force plates using LabView. Videos were then reviewed to ensure the rodent’s hind paw landed on a force panel, which was cross checked with force panel output. If the hind paw was not completely on the force panel or more than one paw landed on an instrumented panel at a time, the trial was excluded. Ten acceptable steps were recorded for each hind leg at each time point for every animal.

Dynamic force data and spatiotemporal data were run through GAITOR Suite codes, as described in Chapter 7. Animal duty factor, temporal symmetry, stride length, step width, and spatial symmetry were calculated from video data, as described in Chapter 6. Additionally, the peak vertical force, time to peak vertical force, and the time between fore and hind peak vertical forces were calculated from force data. All measures were collected for both fore and hind limbs.
Statistical Analysis Comparing Sham to DMM and Both Groups to Expected Results

For gait parameters known to vary with velocity, a linear regression line was used to describe the expected gait parameter value for either male or female sham animals at a given velocity and body weight. These equations were used to predict an expected value for male and female sham mice. Then, for each trial, residuals were calculated by comparing experimental values to expected values, as described in Chapter 8, Figure 8-3. These trial data were then reduced by calculating an animal median. Finally, DMM and sham surgeries were compared using 2-way ANOVA with interaction ($\alpha = 0.05$). In addition, gait symmetries were compared to an expected value of 0.5 and gait imbalances were compared to an expected value of 0.0 using Bonferroni-corrected student’s t-tests (corrected for 10 comparisons).

Results for Male and Female Mice after DMM Surgery

Male Gait Parameters after DMM

Male DMM mice used higher hind limb duty factors in both limbs (Figure 9-2D, next page, left $p = 0.001$, right $p = 0.037$), as shown by the gray circles (DMM) being above the squares (sham) at each week. Male DMM mice also tended to have higher left fore limb duty factors ($p = 0.059$), though this change is only near significance. No differences were seen in either fore limb or hind limb temporal symmetry.
Figure 9-2. Temporal results are shown for male C57/Bl6 mice with either a sham surgery (squares) or destabilization of the medial meniscus (DMM, circles). A) Hildebrand plots of the gait cycle at each week. Solid bars represent the average stance time and white bars indicate standard deviations. B-E) Bar plots are mean ± 95% confidence intervals. ^ indicates a near significant difference, while * indicates a significant difference.

Male DMM mice used shorter stride lengths compared to sham animals, as shown by the gray circles (DMM) being consistently lower than squares (sham) in the top right plot in Figure 9-3B (next page, p = 0.007). No differences were found in the fore limb or hind limb step widths.
Figure 9-3. Spatial results are shown for male C57/Bl6 mice with either a sham surgery (squares) or destabilization of the medial meniscus (DMM, circles). A) Representative plots of the gait cycle at each week. Width and height of the ellipses indicate standard deviation in the stride length and step width, respectively. B-D) Bar plots are mean ± 95% confidence intervals. * indicates a significant difference.
Fore limb peak vertical forces were lower in DMM mice compared to sham mice, as shown by the gray circles (DMM) being consistently lower than squares (sham) in Figure 9-4B (left $p = 0.001$, right $p = 0.029$). For hind limbs, left limb loading in male DMM mice was lower than in sham, as shown by the left gray circles (DMM) being consistently lower than squares (sham) in Figure 9-4D ($p = 0.010$). No differences were seen in the timing of peak vertical force events.

Figure 9-4. Dynamic results are shown for male C57/Bi6 mice with either a sham surgery (squares) or destabilization of the medial meniscus (DMM, circles). A) Representative force curves at each week. B-F) Bar plots are mean ± 95% confidence intervals. * indicates a significant difference.
Female Gait Parameters after DMM

For the hind limbs, female DMM animals walked with lower hind limb duty factors compared to female sham animals, as shown by the gray circles (DMM) being consistently lower than squares (sham) in Figure 9-5D (left p = 0.045, right p = 0.001). Female DMM animals also had higher temporal symmetry of the hind limbs at week 12 (p = 0.005). In the fore limbs, female sham animals used significantly imbalanced fore limb duty factors at week 2, as represented in Figure 9-5B (p = 0.039).

Figure 9-5. Temporal results are shown for female C57/Bl6 mice with either a sham surgery (squares) or destabilization of the medial meniscus (DMM, circles). A) Hildebrand plots of the gait cycle at each week. Solid bars represent the average stance time and white bars indicate standard deviations. B-E) Bar plots are mean ± 95% confidence intervals. * indicates a significant difference.
No significant differences were seen in the stride lengths of female DMM and sham animals (Figure 9-6). Narrower step widths were found in the fore limbs of DMM animals compared to sham animals ($p = 0.006$). No differences were seen in the hind step widths of female DMM and sham animals.

Figure 9-6. Spatial results are shown for female C57/Bl6 mice with either a sham surgery (squares) or destabilization of the medial meniscus (DMM, circles). A) Representative plots of the gait cycle at each week. Width and height of the ellipses indicate standard deviation in the stride length and step width, respectively. B-D) Bar plots are mean ± 95% confidence intervals. * indicates a significant difference.
As shown in Figure 9-7D, female DMM mice loaded the right hind limb (injured) more than sham animals (p = 0.039). However, female DMM mice also took longer to load the right hind limb (injured) (p = 0.004). In the fore limbs, female DMM animals tended to have higher peak vertical forces in the right fore limbs, compared to sham animals (p = 0.061). DMM mice also tended to load the right fore limb more compared to the left fore limb (p = 0.009, Bonferroni correction requires p < 0.005). Additionally, female DMM mice took less time to load the left fore limb compared to shams (p = 0.003).

Figure 9-7. Dynamic results are shown for female C57/Bl6 mice with either a sham surgery (squares) or destabilization of the medial meniscus (DMM, circles). A) Representative force curves at each week. B-F) Bar plots are mean ± 95% confidence intervals. ^ indicates a near significant difference, while * indicates a significant difference.
Discussion of DMM Gait Compensations

This chapter successfully used the EDGAR platform to characterize different gait compensations in male and female mice after destabilization of the medial meniscus. Interestingly, male and female mice used different gait compensations following a destabilized medial meniscus surgery. Male DMM mice developed higher hind limb duty factors, indicating more time spent on both hind limbs in stance. This compensation effectively lowers the single limb support phase of both hind limbs. Additionally, male DMM mice had shorter stride lengths, but relatively normal temporal and spatial symmetries. Male DMM mice also had lower peak vertical forces on both hind limbs. Combined, these factors indicate male animals are bilaterally compensating after DMM surgery through a shuffle-step compensation. Shuffle-stepping is a protective gait characterized by longer stance times which may decrease pain levels by reducing the periods of time where either leg must support load without contralateral support. Though non-significant, this compensatory loading pattern may be further confirmed by the tendency for male DMM mice to have shorter time differences between fore and hind peaks compared to sham animals at weeks 9 and 12 (see Figure 9-4).

Instead of raising hind limb duty factors on both hind limbs, female DMM mice lowered hind limb duty factors, particularly in the right hind limb (injured) (Figure 9-5). Additionally, female DMM mice placed more load on the right hind limb (injured) than sham animals, but also took longer to load the injured limb. Combined, these data are indicative of a steppage gait compensation, also known as “high stepping” and “toe walking”. When seen in humans, the injured foot loses the ability to dorsiflex and the
The limb must be lifted higher to clear the ground; then, the limb is brought quickly down and loaded on the ground.

Paralleling the hind limbs, the right fore limb of female DMM mice experienced higher loading relative to shams, and female DMM mice to took longer to load the left fore limb. In addition, female DMM mice used narrower fore limb step widths. Again, these changes are indicative of a steppage gait.

Given the repeatability of the DMM surgical model and its relevance to clinical osteoarthritis, many studies have examined the biological and behavioral outcomes in DMM mice\textsuperscript{279–281}. Interestingly, prior studies have shown male and female mice respond differently to DMM in terms of histological changes. In particular, joint degeneration was found to be more severe in male mice compared to females, and testosterone was related to osteoarthritis severity\textsuperscript{282}. These results are interesting, especially given the 2016 National Institutes of Health policy regarding gender as an important consideration for preclinical models\textsuperscript{283}. This chapter confirmed functional differences in male and female mice after DMM surgery. Developing different gait compensations following the same destabilization of the joint implies a biological or anatomical factor which causes males to compensate differently than females. However, these changes may also be indicative of the severity of joint degeneration, as male mice tend to have more severe degeneration following DMM\textsuperscript{282}.

To be clear, it is not clear why female mice would select a steppage compensation, while male mice choose to shuffle-step. In humans, steppage gait patterns are typically seen in neurological pathologies\textsuperscript{284}, while shuffle-stepping is common in late stage osteoarthritis and degenerative disc disease. Shuffle-step
Compensations have been previously reported for male rats with a medial meniscus transection\textsuperscript{189,190}. The male mice in this study developed a similar gait profile as male rats with a comparable injury. At this point, it is not clear what benefit a steppage gait pattern has for female mice with a destabilized medial meniscus or whether this compensation is unique to female mice. However, these gender differences certainly warrant further exploration, and future studies should continue to include both male and female animals to further parse out the differences between genders in relation to osteoarthritis.

**Conclusions for the Mouse DMM Model of Knee Osteoarthritis**

Our data confirm the EDGAR platform is able to collect gait data for mice, while describing important differences between male and female mice after DMM surgery. Male mice developed a shuffle-step gait pattern, while female mice developed a steppage gait pattern. The reasons for these differences are currently unknown; however, these gender differences may be particularly important to how male and female animals respond to orthopaedic injuries.
Multiscale biomechanics is a vast field of interdisciplinary research which includes the study of muscles, joints, soft tissue, cartilage, and cells. The term multiscale mechanics refers to the combination of these research areas with the goal of understanding how changes at one level affect other levels.

Two major challenges in multiscale mechanics were addressed in this dissertation. Part 1 addressed mechanical testing of biological tissues, specifically examining the knee meniscus. The effects of hydrating the meniscus in synovial fluid versus saline were evaluated, concluding no differences in the mean results of tension and compression testing. However, performing compression testing in synovial fluid lowers the variance of the data, even though synovial fluid is a far more heterogeneous medium compared to phosphate buffered saline. Secondly, the effects of decellularization and laser drilling were evaluated as a potential method for engineering a meniscus replacement. This study concluded that, while laser drilling statistically lowered some mechanical properties, these properties remained within a physiologic range. Thus, potential may exist for a laser drilled meniscus replacement which largely maintains the complex fiber structure and loading capabilities of the native meniscus, while encouraging vascular ingrowth to the tissue for long term survival.

Part 2 of this dissertation addressed preclinical research methods for osteoarthritis research, specifically evaluating rodent gait. A complete quadrupedal gait characterization was developed to evaluate the effects of low dose monoiodoacetate (MIA) injections, which caused mild joint degeneration. This study concluded low dose
MIA injections resulted in a shuffle-step gait compensation, similar to a previously reported medial meniscus transection model. Secondly, quadrupedal gait characterization was used to evaluate gender differences in mice following destabilization of the medical meniscus. This study concluded male mice with a destabilized meniscus developed a shuffle-step, while female mice developed a steppage gait.

While these studies have not yet combined the full scale of multiscale mechanics, the work in this dissertation has addressed two challenges in tissue mechanics and gait mechanics, which will help to advance the field toward a combined multiscale biomechanics approach in the future. Moreover, the philosophy of scaling mechanics approaches to preclinical tests where biological and scale advantages exist will continue to advance the field.
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LIST OF REFERENCES


37. Athanasiou KA, Sanchez-Adams J. Engineering the Knee Meniscus. 2009.


199. Parvathy SS, Masocha W. Gait analysis of C57BL/6 mice with complete Freund’s adjuvant-induced arthritis using the CatWalk system. BMC Musculoskeletal Disord 2013;14:1–9.


265. Roehmilt ML, Gardner-Morse M, Rowell C, Beynnon BD, Badger GJ. Gait
alterations in rats following attachment of a device and application of altered knee

266. Skøtt Gregersen L, Røslaend T, Arendt-Nielsen L, Whiteside G, Hummel M.
Unrestricted weight bearing as a method for assessment of nociceptive behavior

267. Rashid MH, Theberge Y, Elmes SJ, Perkins MN, McIntosh F. Pharmacological
validation of early and late phase of rat mono-iodoacetate model using the

268. Bagi CM, Zakur DE, Berryman E, Andresen CJ, Wilkie D. Correlation between
μCT imaging, histology and functional capacity of the osteoarthritic knee in the rat

269. Centers for Disease Control and Prevention. Prevalence and most common

Estimates of the prevalence of arthritis and other rheumatic conditions in the

OARSI recommendations for the management of hip and knee osteoarthritis, Part
I: Critical appraisal of existing treatment guidelines and systematic review of

OARSI recommendations for the management of hip and knee osteoarthritis, Part
II: OARSI evidence-based, expert consensus guidelines. Osteoarthritis Cartilage

OARSI recommendations for the management of hip and knee osteoarthritis. Part
III: Changes in evidence following systematic cumulative update of research

274. Oestergaard S, Chouinard L, Doyle N, Karsdal M a., Smith SY, Qvist P, et al. The
utility of measuring C-terminal telopeptides of collagen type II (CTX-II) in serum
and synovial fluid samples for estimation of articular cartilage status in
experimental models of destructive joint diseases. Osteoarthritis Cartilage 2006;14:670–
9.

sensory innervation in monoiodoacetate-induced osteoarthritis in rat knees that
gradually develops neuronal injury in addition to inflammatory pain. BMC

276. Lakes EH, Allen KD. Gait analysis methods for rodent models of arthritic


BIOGRAPHICAL SKETCH

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