Identifying Origins and Consequences of Muscle Dysfunction
After Anterior Cruciate Ligament Injury

by

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Dedication

This one is for my parents. I feel so blessed to be yours. There are no words to describe how thankful I am for the level of support you have provided to me, and equally as important, all I have learned from you. You have taught me to fall in love with becoming the best version of myself, and that character and integrity are invaluable. You have taught me how to advocate for those who don't have a voice, speak up against wrongdoing, and to stand tall in the face of adversity. You have taught me to embrace challenges as opportunities. You have taught me the power of love, forgiveness, accountability, trust, and resilience. Above all, in my opinion, you have shown me that the highest form of love is when all of these things occur in tandem. It is the greatest gift and the biggest blessing. Thank you.

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Abstract

One of the primary targets of post-operative rehabilitation following anterior cruciate ligament reconstruction (ACLR) is quadriceps weakness, in part, given its association with a host of biomechanical strategies that are adopted after ACLR. Together, quadriceps weakness and altered biomechanics contribute to the development of early onset post-traumatic osteoarthritis (PTOA), which has been reported on average in 50% of patients within 10-15 years following ACLR. One predominant theory of PTOA development suggests that quadriceps weakness diminishes the muscle's ability to attenuate shock, leading to altered joint biomechanics and load distribution on articular cartilage. Although quadriceps weakness has been largely attributed to neurological impairments and whole muscle atrophy, these factors do not capture the intrinsic properties of muscle that govern its ability to generate force. Recent studies have started to acknowledge that muscle weakness is part of a multifaceted profile of global muscle dysfunction, encompassing intrinsic properties of muscle such as tissue quality (i.e., composition) and a muscle's mechanical behavior (i.e., fascicle mechanics). However, research on tissue quality and muscle mechanics post-ACLR, is limited, resulting in fundamental gaps in our understanding of their prevalence and functional impact. Thus, the primary goal of this dissertation is to investigate muscle and joint factors that may contribute to early onset PTOA following ACL injury and ACLR. The first study examined the relationships between knee mechanics and subchondral bone architecture in a rodent model with non-invasive ACL injury. The results demonstrated a correlation between knee flexion angle and subchondral bone plate porosity, providing direct evidence linking knee mechanics to early indicators of PTOA development. The second study focused on factors underlying muscle

dysfunction in a chronic cohort of patients with ACLR. Despite having smaller muscles, ACLR patients exhibited between-limb strength, intramuscular fat, and fascicle mechanics (length and angle excursions) that resembled healthy controls. A smaller fascicle angle at peak knee extension moment was also observed in the ACLR limb, which was associated with reduced joint loading, suggesting an inefficiency of the muscle to mechanically adapt to peak loading events. The third study investigated the relationship between muscle size, strength, intramuscular fat and cartilage degeneration using $T_{1\rho}$ and T_2 quantitative imaging. While ACLR patients exhibited comparable between-limb strength and intramuscular fat to healthy controls, muscle size remained smaller in the ACLR knee. In the context of cartilage degeneration, both ACLR and Control groups exhibited between limb differences for various $T_{1\rho}$ and T_2 regions. These differences were not related to quadriceps strength, size, or composition thereby suggesting, other intrinsic properties such as fibrotic tissue development of muscle may be underlying quadriceps dysfunction and contributing to changes in the cartilage matrix following ACLR. In summary, the dissertation findings indicate that altered knee mechanics are related to changes in subchondral bone following ACL injury and that muscle dysfunction may not be fully captured by size and strength assessments alone, emphasizing the important of considering intrinsic muscle properties. While we identified between-limb differences in cartilage matrix, these changes were not related to muscle size, composition, or strength. Future investigations should focus on advancing our understanding of subchondral bone changes in relation to PTOA development and exploring the relationship between muscle's intrinsic properties and function to identify modifiable factors directly linked to the increased risk of early-onset PTOA following ACLR.

1   Introduction & Specific aims

1.1   Statement of the Problem

The current standard of care following an anterior cruciate ligament (ACL) injury is to undergo surgical reconstruction (ACLR) to repair the torn ligament followed by rigorous rehabilitation efforts. While surgery improves joint stability and therapy can enhance physical function, only 20% of patients achieve sufficient quadriceps strength upon completing rehabilitation.[1] Importantly, this strength deficit persists in the long term, even in the absence of direct muscle damage. Quadriceps strength deficits are highly problematic, as they have been linked to alterations in biomechanics, the risk of reinjury,[2] and the development of early onset post-traumatic osteoarthritis (PTOA).[3] Given that the predominant age of injury is young teens[4] and the primary treatment for end-stage PTOA is joint replacement,[5] much work has revolved around understanding the underlying factors contributing to muscle strength deficits following ACLR. Early research has started to uncover that muscle strength is merely one aspect of muscle function, while other intrinsic properties at the biochemical, compositional, and architectural levels may impede muscle recovery following ACLR and be related to early indicators of PTOA development including changes in subchondral bone remodeling and changes in the articular cartilage matrix. These findings emphasize the need for additional investigation in this area.

1.2 Justification of Research

Quadriceps weakness following ACLR has been largely attributed to neurological impairments and gross muscle atrophy.[6-10] However, these factors are largely measured at the whole muscle level and fail to include intrinsic properties of muscle that largely govern force generation and transmission. Additionally, functional impairments have been identified despite quadriceps strength recovery, suggesting strength alone does not fully characterize function. As such, understanding the additional factors that hinder complete recovery and contribute to PTOA after ACLR is of critical importance to pinpoint specific targets for rehabilitation. As such, this dissertation aims to explore intrinsic factors of muscle including architecture and composition that may be compromised following ACLR and contribute to the increased risk for the onset of PTOA development. The factors explored in this dissertation are new, novel, and utilize cutting-edge technology to fully characterize joint mechanics, muscle architecture, composition, and indicators of early PTOA development following ACLR.

1.3   Specific Aims

1.3.1   Aim 1

Aim 1A: Use a non-invasive preclinical model to determine longitudinal changes in knee kinematic profiles following ACL injury

Hypothesis 1A: Knee flexion angle will undergo significant changes in both timing and magnitude throughout the gait cycle post-ACL injury.

Aim 1B: Use a non-invasive preclinical model to determine the associations between knee flexion angle and subchondral bone remodeling, as measured by an increase in porosity.

Hypothesis 1B: Reduced knee flexion angle will be associated with an increase in porosity in the femoral and tibial load bearing regions.

Significance of Aim 1: The majority of previous research utilizing animal models has predominantly employed ACL transection to study ACL injury and PTOA. Importantly, transection models lack in clinical translation as they are invasive and therefore, induce a cascade of negative neuromuscular events that confound observations. For this reason, non-invasive models ACL injury models that induce closed-joint injury through ligament overload have been developed to replicate the human condition more closely. These models are particularly advantageous to fully characterizing disease progression without the confounding effects of surgical transection. As such, the goal of this aim was to investigate biomechanical factors and subchondral bone architecture in a pre-clinical rodent model with

non-invasive ACL injury to understand the relationship between knee mechanics and early

indicators of PTOA following ACL injury.

1.3.2 Aim 2

Aim 2A: Determine if those with a history of ACLR exhibit altered fascicle excursions during walking relative to the uninjured limb and healthy controls.

Hypothesis 2A: Those with a history of ACLR will undergo less fascicle length and angle excursions.

Aim 2B: Determine if intramuscular fat is associated with fascicle length and angle excursions.

Hypothesis 2B: Those with a history of ACLR will have greater intramuscular fat (e.g., fat fraction) and that will be associated with fascicle excursions.

Aim 2C: Determine the associations between fascicle and knee mechanics.

Hypothesis 2C: Fascicles that undergo less excursion, or reduction in length or angle will be associated with a decrease in joint loading measured by peak knee extension moment, rate of moment development, peak power absorption, and knee flexion angle excursion.

Significance of Aim 2: Most commonly, quadriceps function has been measured on global levels including strength, volume, and cross-sectional area following ACLR. However, these measurements are all performed in a static state and fail to evaluate muscle during dynamic activities. Recent advancements in B-mode ultrasound have enabled the assessment of quadriceps fascicle mechanics during movement with early evidence to support that fascicles behave differently than healthy limbs. More specifically, in the acute phases of ACLR recovery, it has been observed that the fascicles in the ACLR limb exhibited eccentric lengthening,

whereas healthy limbs typically show concentric shortening. It remains unknown if fascicle mechanics exhibit similar behavior in the more chronic stages of recovery. Interestingly, a factor that may influence fascicle mechanics in more chronic time points following ACLR is the accumulation of non-contractile elements such as intramuscular fat. Following ACL injury, elevated quantities of fibro-adipogenic progenitors (FAPs) have been identified in the ACLR limb, which are precursors to the development of fat and fibrotic tissue. In addition, preliminary data has shown increases in fat accumulation through an increase in hypoechoic signaling via B-mode ultrasound imaging. However, magnetic resonance imaging (MRI) is a more sensitive measurement of intramuscular fat and enables the evaluation of intramuscular fat in three-dimensions. As there is limited research focused on fascicle mechanics following ACLR and no research to date has investigated muscle fat infiltration following ACLR using MRI, this aim is designed to understand if fascicle mechanics and intramuscular fat are affected in those with a chronic history of ACLR and to determine their relationship with knee mechanics.

1.3.3 Aim 3

Aim 3A: Characterize vasti intramuscular fat both globally and regionally following ACLR

Hypothesis 3A: Those with a history of ACLR would have an increase in intramuscular fat (i.e., higher fat fraction) in the vasti muscles that will be deposited in different regions and disproportionately throughout the muscle.

Aim 3B: Determine if vasti intramuscular fat and fat-cleared muscle volume are predictive of isometric strength.

Hypothesis 3B: Decreased intramuscular fat (i.e., lower fat fraction) and an increased fat-cleared muscle volume would be associated with increased strength.

Aim 3C: Determine if those with a history of ACLR exhibit longer $T_{1\rho}$ and T_2 relaxation times in the tibiofemoral and patellofemoral joint relative to the uninjured limb and healthy controls.

Hypothesis 3C: Those with a history of ACLR will have worse degenerative cartilage outcomes quantified by longer $T_{1\rho}$ and T_2 relaxation times in the ACLR knee thereby, indicating a reduction in proteoglycan density, a disorganization in the type II collagen matrix, and an increase in water content.

Aim 3D: Determine if vasti intramuscular fat, fat-cleared volume, or strength are predictive of $T_{1\rho}$ and/or T_2 relaxation times in those with a history of ACLR.

Hypothesis 3D: Increased intramuscular fat (i.e., higher fat fraction), smaller muscle volumes, and weaker muscles will be predictive of increased $T_{1\rho}/T_2$ relaxation times in those with a history of ACLR.

Significance of Aim 3: Following ACLR, patients are at risk for developing early onset PTOA. While PTOA has largely been attributed to quadriceps strength deficits, data directly linking quadriceps strength to PTOA is sparse. It is plausible that there are alterations in the quality of muscle (e.g., muscle composition) such as an infiltration of intramuscular fat following ACLR, which has been shown in other pathological populations including idiopathic osteoarthritis. However, intramuscular fat of the vasti muscles has not been investigated utilizing a comprehensive technique like quantitative MRI following ACLR. As such, this study is the first to the relationship between muscle size, composition, and function (e.g., strength) in relation to early indicators to PTOA development that would reside in the cartilage matrix.

1.4   Organization of Dissertation

This dissertation has been structured in such a manner that the core chapters (**Chapters 3-5**) detail three separate aims mentioned above in a manuscript format. **Chapter 3** has already been published, and we are in the process of submitting manuscripts for the last two aims (**Chapters 4-5**). The literature review (**Chapter 2**) provides a background and rationale for the dissertation. **Chapter 3** was conducted in a non-invasive rodent model of ACL injury to understand the association between knee mechanics and subchondral bone architecture at days 7-, 14-, 28-, and 56-days following injury. To compare knee mechanics and subchondral bone between ACL injury and controls, we used an age-matched control cohort that did not receive an injury and underwent assessments of knee mechanics at days 7-, 14-, 28-, and 56-days and a single assessment of subchondral bone at 56-days. **Chapter 4** examined *in vivo* fascicle mechanics and intramuscular fat of the vastus lateralis (VL) in humans with a chronic history of ACLR. Patients underwent two experimental sessions to capture I) quadriceps isometric strength, fascicle, and knee mechanics and II) intramuscular fat fraction that was estimated using a multiecho Dixon method, herein denoted as mDixon. **Chapter 5** utilized the same cohort from Chapter 4. Patients underwent the same protocol and in addition to the mDixon, advanced quantitative cartilage imaging was performed to capture protegolocyan density, collagen type II, and water content via $T_{1\rho}$ and T_2 relaxation times. **Chapter 6** summarizes the main findings of chapters 3-5, acknowledges the limitations of each chapter, and outlines future directions for related research.

2   Literature Review

2.1   Background on Anterior Cruciate Ligament (ACL) Injury

Anterior cruciate ligament (ACL) injuries are one of the most common lower extremity injuries[11] with increasing incidence rates over the past 2 decades.[12,13] With approximately 250,000 ACL injuries occurring each year and an average cost of $11,000 per injury,[12] the annual health care expenditure for ACL injuries in the United States is close to $2 billion.[14,15] Following ACL injury, patients generally opt to undergo ACL reconstruction (ACLR) to restore the mechanical stability of the joint followed by rigorous and prolonged rehabilitation primarily aimed regaining range of motion, strength, neuromuscular control, and returning patients to physical activity. Despite such efforts, patients experience a myriad of impairments affecting their quality of life, physical function, and return to activity.[16] Importantly, patients have an increased risk of re-injury[2,17-19] and early onset post-traumatic osteoarthritis (PTOA),[3] which has been reported on average in 50% of patients within 10-15 years following ACLR.[3] Notably, PTOA is associated with increased pain,[20] a poorer quality of life,[20,21] and extensive functional deficits.[20,21] Given the high societal and economic burden, it is critical that we understand the factors that contribute to poor physical function and the development of PTOA following ACL injury.

Quadriceps weakness is a major impairment after ACL injury and ACLR as it affects knee related quality of life[22] and reduces physical function.[23] Additionally, quadriceps weakness is considered to contribute to a host of biomechanical strategies adapted after ACL injury and ACLR that increase load on articular cartilage in the knee, and increases the risk of developing knee

PTOA.[24] As such, quadriceps strength is often targeted during rehabilitation due its modifiability and clinical importance. However, despite undergoing extensive rehabilitation, only 1 in 5 patients are able to regain adequate strength levels in their ACL limb at time to return to sport.[1] It has been shown that these impairments persist for years with reports of strength deficits up to 15 years following ACL injury.[23,25,26] These findings highlight the muscle's impaired ability to respond to rehabilitation efforts, leading researchers to investigate the origins of quadriceps weakness. Over the past twenty years, quadriceps weakness has been largely blamed on neurological impairments and whole muscle atrophy.[27,28] While important, atrophy, or a loss in muscle volume, is a simplified measure that does not reflect the quality of muscle tissue or the intrinsic properties of muscle that govern force production. Recent work has started to acknowledge that muscle weakness falls under a larger multifaceted profile of muscle dysfunction, observed by changes in tissue quality (i.e., composition, architecture, and functional behavior).[29] As tissue quality has been much less studied following ACL injury and reconstruction, fundamental gaps exists in our understanding of its prevalence, functional impact, and clinical importance. As such, this dissertation aims to comprehensively describe muscular function through assessments of both the quantity (e.g., volume) and quality (e.g., composition, architecture, functional behavior) of muscle and understanding how these factors are associated with PTOA following ACLR. The following sections provide an overview and contextual background of a topic followed by the findings and potential implications following ACL injury and reconstruction.

2.2 Conventional Pathways of Quadriceps Dysfunction After ACL Injury and Reconstruction

Traditionally, quadriceps dysfunction has been attributed to two main mechanisms: neurological impairments and atrophy (e.g., volumetric muscle loss) as discussed in the sections below.

2.2.1 Neurological Impairments

In addition to ACL injuries compromising static joint stability, ACL injuries also induce muscle inhibition, or the inability to fully activate the quadriceps muscle. Muscle inhibition is ultimately due to the suboptimal recruitment and/or firing of alpha motor neurons but the primary neural pathways that dictate alpha motor neuron activity are numerous.[6] In the acute stages of injury, there is a loss of afferent signaling to the central nervous system due to the physical loss of the ligament and its mechanoreceptors,[7] and a host of peripheral factors that erupt in the natural response to injury including joint inflammation, effusion,[30,31] and pain.[7,30] Early changes in afferent signaling are thought to influence the centrally-mediated mechanisms of muscle activation (e.g., spinal-reflexive pathways), thereby causing inhibition of the quadriceps motor neuron pool that innervate the muscle and are required for contraction.[8] The centrally-mediated mechanisms of muscle activation also include supraspinal pathways (e.g., corticospinal) that have been shown to primarily be affected in the later stages of rehabiliation.[6,8] It would be expected that the reported neurological impairments at centrally-mediated levels would recover after the peripheral contributions subside (e.g., inflammation, effusion, pain), however, alterations in neural excitability have been reportedly in the early and late stages following ACLR.[8] While the reported neurological impairments have primarily originated from the spinal-reflexive and corticospinal pathways, recognition to adaptations effecting the sensorimotor and visual-motor systems that

underpin motor control have also been reported.[32] Ultimately, ACL injury alters neural input required for proper signaling between spinal and supraspinal centers that underlie the patient's inability to fully contract their muscle.

2.2.2 Volumetric Measures of Quadriceps Atrophy

Following ACL injury and ACLR, patients experience a widespread loss of quadriceps muscle size through a reduction in muscle volume and cross sectional area (CSA).[10] Recently, Birchmeier[9] and Dutaillis et al[10] performed comprehensive systematic reviews of muscle size following ACL injury and ACLR. Data from these reports provide evidence of size reductions across all quadriceps muscles but primarily the vastii (lateralis, medialis, and intermedius).[10] Interestingly, Birchmeier et al[9] found that only a small portion of quadriceps weakness could be explained by quadriceps atrophy and pointed to size reductions in concert with muscle structure and composition that may help to better elucidate the protracted quadriceps weakness following ACLR.[9] Nonetheless, evidence of muscle size reductions due to a loss of muscle volume and/or CSA have been well described.[33]

To maintain muscle volume, a balance of muscle protein synthesis to muscle degradation is required. In healthy populations, these pathways are responsive to physical activity that result in muscle volume loss or growth, as a patient increases or decreases levels of activity. For example, with disuse atrophy, there is a reduction in muscle fiber size, measured by its CSA, which leads to a decline in the force generating capacity of the muscle thereby, contributing to muscle weakness.[34] However, in healthy persons, disuse atrophy resolves with the re-introduction of physical activity. Interestingly, following ACLR, the re-introduction of physical activity does not resolve early atrophic changes, suggesting that the muscle's response differs than that of a healthy person with disuse atrophy.[35,36] Therefore, exploring factors that regulate muscle size and how these factors

are impacted following ACL injury and reconstruction seems necessary to identify therapeutic strategies to promote muscle growth.

2.2.3 Summary of Conventional Pathways of Quadriceps Dysfunction

While findings of neurological impairments and prolonged atrophy help characterize the protracted muscle dysfunction after ACL injury and reconstruction, they are reflective of whole-muscle properties (volume, gross muscle activation) and disregard the intrinsic properties of a muscle's structure and composition that are important for muscle function and force production. To this point, maladaptive tissue properties have been reported on cellular (e.g., molecular signaling cascades), compositional (e.g., fiber typing, fibrotic tissue development), and functional levels (e.g., muscle architecture and contractile function) following ACL injury that suggest a local degenerative process affecting both tissue quality and dynamic muscle behavior.[37-41] While less recognized, these muscle adaptations have significant roles in muscle function and thus, warrant further consideration as to how these pathways may contribute to the experienced muscle dysfunction following ACL injury. Together with atrophy and inhibition, these less recognized pathways paint a compelling story into an intricately intertwined and broadened circuitry of neuromuscular function beyond what is traditionally appreciated. Better understanding the roles of these less recognized pathways may help elucidate factors of quadriceps dysfunction that can be targeted with concentrated rehabilitation efforts to prevent or delay the development of early PTOA as described in the sections below.

2.3 Non-Conventional Pathways of Quadriceps Dysfunction

2.3.1 Skeletal Muscle Structure and Composition Overview

Skeletal muscle is composed of muscle fibers, extracellular matrix (ECM), blood capillaries and nerves that supply the muscle. Muscle fibers are the contractile elements of the muscle that comprise a muscle fascicle. Muscle fibers are characterized by protein isoforms, or genetic make-up, that determine its structure and functional capacities. Type 1 slow-twitch fibers are fatigue resistant and primarily contribute to prolonged, low force and speed contractions, whereas type II fast-twitch fibers are more fatigable and primarily contribute to high force and speed contractions. Given these properties, type 1 fibers are generally seen in endurance-based activities like marathon running, whereas type II fibers are generally seen in high velocity-based activities like sprinting and are further subdivided into type IIa and IIb or IIx fibers that differ by their oxidative capacity (i.e., IIa are oxidative and IIb/IIx are glycolytic). Interestingly, muscle fibers adapt in response to activity meaning that functional demands can cause a fiber type transition.[42] For instance, changes in mechanical loading or neural stimuli can initiate a fiber type transition from slow-to-fast or fast-to-slow.[43] There are other occurrences that can change fiber types like denervation and re-innervation events that commonly occur in nerve injuries and aging.[43,44] In general, fiber type transitions represent a change in functional capacity (e.g., force, power, velocity) which can have negative implications for training and rehabilitation and may impact quadriceps function following ACL injury.[42]

The ECM is a mesh-like framework composed of non-contractile elements (e.g., collagen, glycoproteins, proteoglycans, and elastin) that form a supportive environment for muscle fibers and neuromuscular junctions.[45] Muscle structure (i.e., architecture) and composition (i.e., ECM) play pivotal roles in the generation and transmittance of force during muscle contractions. While

less studied following ACL injury, the presence of injury and disease can induce alterations in the structure and composition of muscle that interfere with the interactions of contractile and non-contractile elements and may be underlying muscle dysfunction as discussed in the sections below.

2.3.1.1 Skeletal Muscle Architecture

Skeletal muscle is a highly organized structure that largely dictates total force and velocity potentials.[46,47] A muscle's architecture is largely characterized by fascicle length and pennation angle that undergo structural changes in response to injury,[48] immobilization,[49] and training.[49] Fascicle length refers to the number of sarcomeres in series and primarily dictates fascicle velocity and excursion. While fascicle length adaptations (e.g., shortening or lengthening) in response to training vary,[50-53] fascicles most often shorten in response to injury[54] and immobilization,[55] which is predominantly thought to reflect a loss of sarcomeres in series. This is clinically relevant as shorter fascicles operate in a smaller working range and can increase the risk of strain injury while also limiting force production.[56] Pennation angle is another important architectural property of muscle that refers to the arrangement of fascicles relative to the axis of force production. During dynamic contractions the muscle's pennation angle and fascicle length interact to optimize function (e.g., force and velocity). This tradeoff between pennation angle and fascicle length is a property called architectural gear ratio or AGR. AGR reflects the ability of a muscle to shape change during movement, while fibers rotate in and out of the muscle's line of action to regulate how much force is transmitted throughout muscle fibers and to the muscle-tendon. As a muscle's volume remains constant during movement, the generation and transmittance of force in different directions relative to a muscle's line of action make the muscle favorable to either a high force or high velocity contraction by the shape changes that occur. Importantly, the ability to shape change is lost with aging[57] and in part, may be due to an increase in connective tissue stiffness[57] or

accumulation of connective tissue elements that occur in cases of fibrosis[58,59] and may restrict muscle bulging (e.g., thickness or width) and limit fiber rotation required for an efficient transmission of force during movement.[58] Understanding how these architectural properties are impacted following ACL injury and reconstruction may help elucidate factors underlying the persistent muscle dysfunction.

2.3.1.2 Evidence of Structural Alterations Following ACL Injury and Reconstruction

While the clinical importance of these architectural properties is evident, there is limited evidence to support that changes in muscle architecture exist following ACL injury. There have been inconclusive results on changes in fascicle length, pennation angle, and muscle thickness following ACLR.[38,60] Some datasets[38] suggest early changes in pennation angle that are recovered through rehabilitation. Others[60] have reported no changes in pennation angle but a reduction in muscle thickness. Importantly, these assessments were performed in static states (i.e., at rest), giving no insight into how the muscle behaves during movement. Determining the changes in fascicle length, pennation angle, muscle thickness, and the muscle-tendon unit that underlie shape change and force/velocity potentials may help elucidate the changes in muscle composition and architecture that impact muscle function.

Recent advancements in dynamic ultrasound have allowed for the investigation of a muscle's mechanical behavior.[61] This is an important development as previous work highlights muscle atrophy and changes in composition following ACL injury, but have not determined the functional impact of these findings. One of the first studies to explore muscle mechanics investigated fascicle behavior during concentric isokinetic strength testing following ACL reconstruction.[62] Researchers found that muscle fascicles in the injured limbs following ACL reconstruction operate at shorter fascicle lengths, smaller pennation angles, and have a slower

velocity during concentric contractions.[62] Of importance, these researchers did not find a difference in fascicle length or pennation angle in the same cohort at rest, suggesting that static assessments, as performed by previously in ACL patients,[38,60] may mask differences in functional behavior of the muscle.[62] To this point, recent work by Munsch et al[63] performed the first assessment of muscle mechanics during walking and found that vastus lateralis (VL) fascicles in the ACLR limb were unable to resist lengthening during the weight acceptance phase of gait when compared to the contralateral limb. The authors suggest that this unexpected lengthening behavior of the fascicles may be due to a decrease in neural drive, and the inability of the muscle fascicles to operate quasi-isometrically in order to generate significant counter-breaking force during weight acceptance.[63] Independent of the contraction mode (e.g., isometric, concentric, or eccentric), it is apparent that following ACL injury, fascicles exhibit maladaptive behavior compared to uninjured limbs.

2.3.1.3 The Role of The Muscle-Tendon Unit in Muscle Function

The study of muscle architecture and muscle mechanics does not stop at fascicle architecture and behavior. In recent years researchers have started studying behavior of the muscle-tendon unit (MTU) as both the contractile (muscle fascicles) and series elastic elements (tendinous tissue) that influence force and power production and energy dissipation. This is clinically important as a balanced interaction between contractile (muscle fascicles) and series elastic elements (tendinous tissue) is required for optimizing force and movement patterns.[46] However, many methods that investigate the behavior of the MTU as a whole (fascicle and tendon) require a conglomerate of indirect measurement techniques (e.g., predictive estimations). Using this technique, it has been reported that there is a decoupling that occurs between fascicles and the tendons in distal leg muscles (e.g. gastrocnemius, soleus), where length changes primarily occur

in the tendon rather than the muscle, which primarily performs quasi-isometric actions.[46,64-66] Tendon lengthening has been shown to be a protective mechanism to reduce the mechanical strain on fascicles,[67] where the tendon lengthens to a greater extent in order to attenuate power and dissipate force. To date, researchers have remained reliant on the aforementioned predictive estimation methods to determine if this pattern of muscle and tendon decoupling is consistent in the proximal leg muscles (e.g. VL) but have reported inconsistencies due to measurement technique.[46,68,69] Given that MTU as a whole governs force generation and transmission, it remains plausible that alterations at either the fascicle or tendon level would disturb an maximal force-generating interaction between the contractile and series elements of the MTU in the presence of injury and contribute to muscle dysfunction. Early data show that the MTU lengthens less on the ACLR limb than the contralateral limb following ACLR. While valuable, only indirect estimations were made of MTU elongation.[63] Recent technological advances in measurement techniques now permit the simultaneous and direct assessment of fascicle and tendon behavior using B-mode ultrasound, allowing for a comprehensive assessment of fascicle and tendon behavior following ACL injury that may help uncover factors preventing full muscle recovery after injury.

2.3.2   Muscle Fibrosis Overview

The ECM plays a pivotal role in force transmission and the maintenance and repair of muscle fibers when subjected to injury. Force transmission is an intricately intertwined system of interactions between contractile (i.e., actin and myosin) and non-contractile (ECM) components of muscle. Force is known to be predominantly generated through fiber shortening that is then transmitted longitudinally through interactions between muscle fibers and collagen within the ECM to the tendon.[70] However, a new understanding of muscle structure has revealed that muscle is much more interconnected than we previously thought with branches that split off and connect

myofibrils.[71] This mesh-like framework of muscle enables force to be transmitted across the width of the muscle. The significance of this, is that any change to this environment (like a build-up of ECM or fatty tissue infiltration) has the potential to disrupt cellular signaling needed for muscle growth and force production during muscle contractions.

An accumulation or buildup of excessive and non-contractile (e.g., fatty infiltration) components of the ECM is called muscle fibrosis. As the ECM provides structural support for many cellular processes and is essential for adequate force transmission, over-accumulation can be prohibitive to both regulatory cellular processes and dynamic contractions. Muscle remodeling requires a coordinated response by inflammatory, fibro-adipogenic progenitors (FAPs), and satellite cells. FAPs and satellite cells differ in origin where satellite cells are myogenic cells for muscle regeneration, FAPs aid in satellite cell differentiation through a network of cellular interactions. In a natural response to injury, fibronectin binds to components of the ECM, to form new, temporary ECM material.[45] This temporary ECM is degraded by proteolytic enzymes and FAPs, a process that prevents excessive ECM depositions and recruits inflammatory and satellite cells.[72,73] However, when signaling between these cells is dysregulated due to injury or disease, muscle fibrosis, can occur.[74] In conditions of fibrosis, FAPs remain elevated, thereby inhibiting satellite cell activity,[75] disrupting ECM remodeling,[76,77] sustaining high-levels of inflammation,[78] and promoting further differentiation to fat and fibrotic tissue.[78] This negative signaling cascade reflects a decline in muscle tissue quality, ECM integrity, and impairs the ability of muscle to respond to rehabilitation efforts through the accumulation of non-contractile elements and factors that negatively impact muscle growth. There is some evidence to support this negative signaling cascade following ACL injury,[37,38,40] but direct assessments of skeletal muscle fibrosis and its functional impact following ACL injury are limited.[40]

Whether by fibrosis or intramuscular fat (IMF), the over-accumulation of maladaptive connective tissue increases muscle stiffness (passive) through compromised gliding between layers of collagenous tissue in the ECM. While this increase in stiffness can sometimes be beneficial for force generation, the over accumulation of connective tissue can also be inhibiting. To this point, an increase in non-contractile elements can develop into localized fatty deposits or streaks that reside throughout the muscle and commonly occurs alongside the reduction of contractile elements that can induce the deformation of muscle structure[79], and restrict force transmission between myofibrils,[70] aponeuroses, and the musculotendinous junctions. These constraints can consequently limit strength recovery.[80-82] Furthermore, the accumulation of ECM components can limit muscle growth and restrict contractile behavior during movement. However, it is currently unknown if the presence of fat or fibrotic tissue functionally impacts the muscle during daily movements following ACL injury.

2.3.2.1 Evidence of Cellular and Morphological Alterations in Fibrotic Tissue Development

Only a handful of studies have investigated cellular and morphological pathways that may underlie fibrotic tissue development following ACL injury and have found buildups of collagen deposits within the ECM of the VL including the interstitial spaces within and surrounding muscle fibers.[37,38] These findings were correlated to increases in fibroblasts and FAPs and occurred alongside increases in transforming growth factor-beta (TGF-ß1), a multifunctional cytokine that is a profibrotic regulator of connective tissue[37] and considered to initiate signaling cascades involved in fibrotic tissue development.[40,83] These findings support that following ACL injury a signaling cascade conducive to fibrotic tissue development is initiated, in part, through TGF-ß1. This provides evidence of intrinsic changes in muscle quality that have been implicated in

quadriceps strength deficits in populations with knee OA[39] and may be underlying the protracted quadriceps dysfunction following ACL injury.[35,40,84]

Another key factor implicated in fibrotic tissue development is myostatin, a negative regulator of muscle mass that is closely related to TGF-ß1.[85] When myostatin is overexpressed, it increases quantities of FAPs pointing to its regulatory role in fibrosis. Following ACL injury, Peck et al[40] reported a positive association between myostatin and fibroblasts. This occurred alongside increased local levels of myostatin and ECM components, collagen, and fibronectin, in the injured limb. Future work should investigate if profibrotic pathways are stimulated due to elevated levels of myostatin following ACL injury.

Collectively, these findings provide evidence to support the dysregulation of cellular and morphological mechanisms that are required for proper muscle function following ACL injury. However, these findings were determined in small cohorts using muscle biopsies. As non-uniformities in tissue compositions and morphology reside across whole muscles,[86] muscle biopsies provide only a narrow representation of the muscle. In addition, it is unknown how these findings functionally impact the muscle. While little evidence is present to truly substantiate claims of muscle fibrosis or the functional impact fibrosis has in the population affected by ACL injury, it remains highly plausible that injury initiates a signaling cascade that enables the entrance of fat and fibrotic tissue and thus, alters contractile function and contributes to the protracted muscle weakness and dysfunction.

2.3.2.2 The Role of Intramuscular Fat in Fibrotic Tissue Development

IMF is a component of the non-contractile elements involved in fibrotic tissue development. As mentioned, the signaling cascades involved in fibrotic tissue development also enables the entrance of IMF. In addition, IMF can occur with muscle disuse or unloading,[87] and

has been associated with aging,[88,89] obesity,[90] insulin insensitivity,[91] bone fractures,[92] cardiovascular disease,[93] and functional deficits including quadriceps weakness in older and osteoarthritic populations.[94-96] In fact, IMF of the VL has been found to be a better predictor of knee OA than muscle size alone,[97,98] supporting the premise that muscle quality is an important factor in muscle function as it pertains to OA. While it is known the environment created through the accumulation of non-contractile elements like fat is not conducive to muscle growth or force generation, it remains unknown if there is an over-accumulation of fat following ACL injury. Using echo intensity measured via B-mode ultrasonography to measure intramuscular fat, Garcia et al[99] found lower values of intramuscular fat of the VL were associated with improved self-reported function following ACLR. While important, echo intensity is a surrogate, 2-dimensional, and indirect measure of intramuscular fat. Advancements in technology permit 3-dimensional assessments using advanced MRI sequences to investigate whole and regional fat deposits that may be present following ACL injury but have not yet been performed in this population. Of importance, how IMF functionally impacts the muscle during dynamic movements or how it may relate to the development of PTOA following ACL injury has not been investigated. Further investigations are warranted to determine the prevalence and functional impact of IMF following ACL injury.

2.3.3   Single Fiber Size, Phenotype, and Contractility

Following ACL injury, mixed results have been reported for fiber type transitions in the VL including atrophy of type I and II fibers,[100] type 1 only,[101] or type IIa only.[38] Noehren et al[38] reported a significant decrease in type IIa that occurred alongside an increase in hybrid type IIa/x following ACLR, supporting a transition to faster fiber types. It remains plausible that the surgery rather than the injury induced the fiber type transition. Regardless of injury or ACLR, the increase

in type IIa/x fibers has been associated with quadriceps atrophy following ACLR and strength deficits in individuals with ACLR and knee osteoarthritis. Given the association between fiber type and quadriceps size and function, understanding the mechanisms of fiber type changes and how to maintain size and type following ACLR has important clinical implications to mitigating and understanding quadriceps dysfunction following ACL injury.

In addition to fiber type transitions, the contractility of single fibers can be assessed using muscle biopsies, which decouples neural activation with muscle fiber function. Using this approach, deficits in isometric force production of type I and type II fibers compared to contralateral and control limbs have been reported following ACLR.[102-104] These findings appeared to occur in concert with fiber-level atrophy (i.e., CSA). In order to determine if atrophy mediated single fiber force production, Tourville et al[102] normalized force by CSA to determine isometric tension and found that there were fiber specific adaptations with only differences in type IIa fibers reaching statistical significance between ACLR and contralateral limbs. This is important as it provides evidence of differences in single fiber contractility of type IIa fibers independent of fiber atrophy. Additionally, deficits in isometric tension and power early after surgery were associated with whole muscle strength deficits and patient-reported outcomes (i.e., pain, function) at 6 months post-ACLR. This differs from findings by Gumucio et al,[104] who found that deficits in isometric tension at the time of ACLR, recovers 6 months post-ACLR. The differences in findings may reflect low sample sizes, and comparisons between ACLR and contralateral limbs or ACLR and control limbs. Regardless of the findings, it is evident changes in single fiber contractility occur independently of neural impairments following ACLR. Better understanding the mechanisms by which these changes in single fiber contractility occur and in relation to whole muscle function

may help elucidate the changes in whole muscle dysfunction following ACL injury and reconstruction and help determine modifiable factors that could be targeted through interventions.

2.4 Articular Cartilage and Post-Traumatic Osteoarthritis (PTOA)

2.4.1 Articular Cartilage

The femur, tibia, and patella are the 3 bones within the knee that articulate to form the tibiofemoral and patellofemoral joints. Articular cartilage lines the surfaces of these joints and functionally serves as a cushion to absorb, distribute and transmit forces.[105] Despite only being 2-4 mm thick, articular cartilage has a remarkable resilience to withstand fluctuating environments of high, variable, and cyclic loads across its lifespan pointing to its unique mechanical properties that enable it to distribute load and minimize stress on surrounding joint tissues.[105] Given that articular cartilage is avascular tissue with a limited capacity to repair itself, understanding how to maintain the integrity of articular cartilage across different loads and conditions is pivotal to preserving joint health.

Articular cartilage is a multiphasic tissue that is characterized by its solid, fluid, and ionic phases, and its ECM that is made up of many macromolecules including collagen, proteoglycan, and numerous other non-collagenous proteins.[106] Each phase and the interaction between phases significantly contributes to cartilage's mechanical and biochemical function. Interstitial water is the most prominent component of articular cartilage making up 60-85% of the cartilage volume.[107] Proteoglycans, collagen, and the surrounding ECM mesh-network form the solid phase, while water and electrolyte ions (e.g., calcium, potassium, chloride, sodium) make up the freely flowing fluid phase. Proteoglycans are composed of core-protein and covalently attached glycosaminoglycan (GAG) chain(s) that provides resistance to compression through aggregating proteins like aggrecan, the most abundant proteoglycan in articular cartilage.[108] Collagen can be

separated by their function in forming fibrils (e.g., types I, II, III, V, XI) or the nonfibrillar network. Type II collagen makes up about 90% of the collagen in articular cartilage with other types (e.g., types VI, IX, X, XI) appearing to a lesser extent.[106] Collagen fibrils are laterally linked helical structures, composed of polypeptide chains, that are covalently cross-linked. It is this structure and arrangement of fibrillar components that provides cartilage with its tensile properties. Proteoglycans form a porous-permeable material intricately linked with collagen that helps regulate the hydration of articular cartilage by governing the balance between cartilage osmotic pressure and tensile force.[107] The positive and negative ion interactions allow for water to move in and out of the tissue, altering osmotic pressure within cartilage that is resisted by a complex proteoglycan-collagen network.[106] Importantly, the interaction between the solid and fluid phases and components gives rise to cartilage's viscoelastic properties that enable it to deform and support load bearing in variety of conditions.[107]

Importantly, chondrocyte cells are the only metabolically active cell within articular cartilage and therefore, are responsible for synthesis and organization of ECM and its constitutes.[106] Chondrocytes only account for about 1-5% of human articular cartilage[109] and their density and arrangement varies through depths of articular cartilage.[106] They are embedded in complex and distinct pericellular matrix that is rich in proteoglycan content and interacts with the surrounding ECM, through an important molecule called integrin, which influences how chondrocytes responds to mechanotransduction and biochemical stimuli (e.g., cell deformation, matrix turnover).[110] As such, chondrocytes are tasked with maintaining a balance between synthesis and degradation of the tissue's ECM complex to sustain healthy function of articular cartilage.[106]
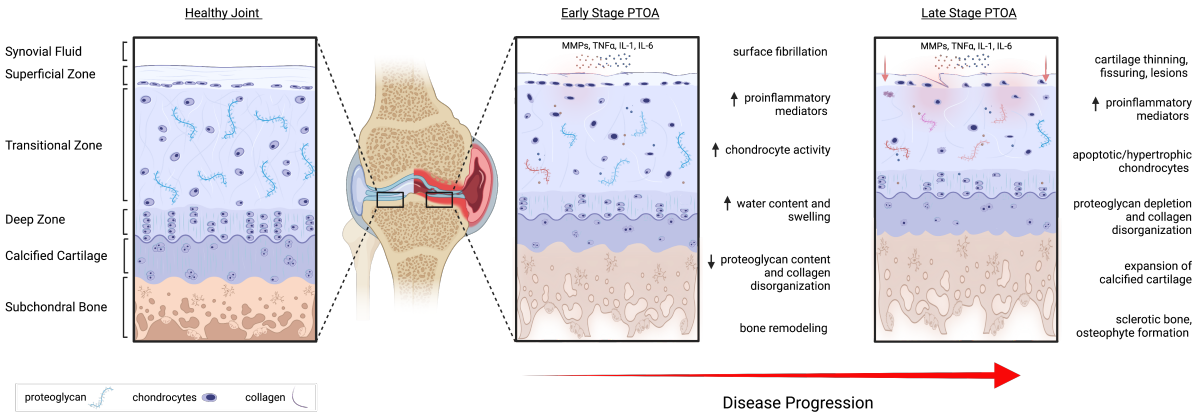
Figure 1: Healthy (left) vs. early (middle) and late (right) degenerative processes of the knee joint that occur with post-traumatic osteoarthritis (PTOA). Early in the degenerative process there are compositional changes of the extracellular matrix (ECM) characterized by surface fibrillation, collagen disorganization, and a decrease in proteoglycan content. The disruption of the ECM alters the biochemical and mechanosensitive processes that enable cartilage to dissipate and adapt to load, resulting in further progression into later stage PTOA. With further progression, chondrocyte cells become apoptotic/hypertrophic, there is thinning and fissuring on the surface membrane, and the subchondral bone becomes sclerotic with osteophyte development. Created with BioRender.com

2.4.2 Articular Cartilage Zonal Properties

Articular cartilage is split zonally based on its compositional structure that gives rise to its unique mechanical properties[105] There are 4 zones of cartilage including the superficial, transitional, deep, and calcified zones that differ in its composition (e.g., collagen, proteoglycans) and structure (e.g., cell density, and collagen orientation) that enable each zone to have unique contributions to maintaining load bearing function or cartilage (Figure 1: left).[111]

The superficial zone is the thinnest zone but it is able to resist tension and some compressive force, in part due to its densely packed collagen fibrils that lie parallel to the joint surface.[106] The chondrocyte cells in the superficial zone primarily lie flat to the surface and are smaller in size but greater in density than other zones, which provides some resistance to shear stress.[112] This zone has the highest concentration of water and lowest concentration of proteoglycans, pointing to its large deformable nature that relies on the dense type II collagen to

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maintain osmotic pressure during loading.[112] In cases where the superficial zone degenerates, collagen fibrillation and disorganization can occur that limit the zone's ability to dampen forces due to a greater fluid exchange and consequently, the zones deep to the superficial zone become subjected to higher amounts of stress.[113]

The transitional zone has a lesser extent of chondrocyte cells that are spherical and lower density than the superficial zone.[111] Collagen is less abundant resulting in less tensile stiffness, but collagen fibrils are more disordered and arranged obliquely in this zone, permitting a greater resistance to shear stresses.[111] There is an increased concentration of proteoglycans in the transitional zone that provide greater compressive stiffness and more resistance to deformation than the superficial zone.[114]

The deep zone has the highest concentration of proteoglycans and lowest water concentration.[111] Chondrocyte cells in this zone are the least abundant and arranged in vertical columns. Similarly, to the transitional zone, the deep zone is rich in proteoglycan content that reflects its high compressive properties.[111] Unlike the superficial and transitional zone, collagen fibrils in the deep zone are oriented perpendicular to the joint surface.[115] The fibrils are also the largest in diameter and cross the calcified cartilage border to insert into the calcified zone, acting like an anchor between the cartilage tissue and subchondral bone.[106] Given the perpendicular orientation of collagen fibrils, this zone has less resistance to tensile and shear stresses than the superficial and deep zones.[106]Alterations

2.4.3   Alterations in Knee Joint Structure and Function with PTOA

PTOA is a whole joint disease that can be characterized by joint space narrowing, osteophyte development, subchondral bone remodeling, and articular cartilage breakdown (Figure 1). While the pathogenesis of PTOA differs from idiopathic osteoarthritis, which occurs without

joint injury, both diseases may ultimately result in knee arthroplasty given the limited treatment options once the disease presents.[116] Therefore, understanding the pathogenesis of PTOA may help elucidate early windows of opportunity for intervention and factors that can respond to targeted rehabilitation efforts.

Compositional changes affecting water concentration, proteoglycan content, and matrix organization precede structural changes (e.g., joint space narrowing, osteophyte development) that occur in the later stages of PTOA progression (Figure 1). The cartilage is subjected to compressive, tensile, and shear forces during loading that help explain its flow-dependent and flow-independent properties. Flow-dependent properties are largely driven by the ability of proteoglycan to pressurize interstitial fluid and the tissue's permeability that define the time-dependent responses under load (stress and strain behaviors), whereas flow-independent properties are driven by the proteoglycan-collagen solid matrix complex that resists tensile and shear stresses.[107] Under a constant load, cartilage undergoes a rapid deformation to exude interstitial fluid that is followed by a gradual decrease in deformation due to the increased strain and reliance on the solid matrix. As such, the compositional components (e.g., interstitial water, proteoglycan, and collagen) and their interactions govern the viscoelastic properties and the ability of cartilage to resist deformation during joint loading.

In early stages of PTOA, significant changes in composition can reduce the ability of cartilage to dissipate load through altering tissue permeability and the ability of proteoglycans to pressurize the tissue and maintain its compressive stiffness (Figure 1: middle).[117,118] These changes are largely characterized by chondrocyte deformation, decreases in proteoglycan content, collagen disruption, and increases in interstitial fluid,[119] which have been shown in pre-clinical models of ACL transection.[117,118,120,121] Importantly these changes in composition occurred in conjunction

with a decrease in compressive stiffness.[117,118] Over time, this can place undue stress on the solid matrix and lead to further degradation depicted by collagen disorganization, osteophyte development, and surface fissuring seen in later staged PTOA (Figure 1: right).[122,123] The damage to composition in the early posttraumatic phase due to ACL injury is also accompanied with an influx of inflammatory markers and changes in biochemical processes that are required to maintain joint homeostasis.[114,116] Following ACL injury[124,125] and ACLR,[126] processes that are responsible for regulating cartilage metabolism (e.g., chondrocyte activity) through the balance of anabolic and catabolic pathways are disrupted.[114] For instance, matrix metalloproteinases that are involved in cartilage degradation are elevated following ACL injury[127] and ACLR. In addition, increases in proinflammatory cytokines (e.g., tumor necrosis factor alpha, interleukin-1, interleukin-6) and proinflammatory mediators (e.g., nitric oxide) after ACL injury[124,126] and ACLR have been reported. Overall, the changes in biochemical signaling interfere with joint homeostasis and change how chondrocytes sense and respond to stimuli, which can further contribute to compositional changes in the ECM.[114,128-130]

While changes in cartilage metabolism, structure, and composition largely characterize PTOA, the changes in cellular signaling and loading also effect subchondral bone. Early changes in subchondral bone are also considered markers of PTOA and have been commonly reported in cases of idiopathic osteoarthritis.[131,132] Damage to subchondral bone can occur due to the injury itself or without any direct insult, and present as a bone bruise, fracture or changes in bone remodeling.[114,133,134] It has been suggested that alterations in subchondral bone can diminish the ability of the joint to withstand and distribute forces, which would increase stress on the underlying articular cartilage.[135] And furthermore, that damage to subchondral bone can also initiate cartilage degradation through bone and cartilage cross-talk of biomarkers like proinflammatory cytokines

that can occur with bone remodeling[136] and ACL injury or ACLR.[137] Therefore, understanding the relationship between subchondral bone and articular cartilage may help in our understanding of the pathogenesis of PTOA.

Despite that the specific role of subchondral bone in PTOA onset and progression has not been elucidated, it has been shown that subchondral bone remodeling (e.g., osteophyte development, bone resorption) can occur prior to[134] or concurrently with[138,139] damage of articular cartilage following ACL injury. Birch et al[140] also reported changes in subchondral trabecular bone architecture following ACL injury and suggested that it may be representative of a stress-shielding mechanism induced by redistribution of contact stress in the tibiofemoral joint that could cause thickening or narrowing of trabeculae.[140,141] Others[142] have found a thickening of the femoral subchondral bone plate in the absence of cartilage thickness changes following ACLR, which is consistent with findings of subchondral bone thickening in idiopathic osteoarthritis.[143] One of the challenges in understanding the relationship between subchondral bone remodeling and articular cartilage degeneration and the longitudinal manifestation of them with PTOA is the reliance on MRI techniques for the quantification of bone microarchitecture and articular cartilage that endure high costs and require patients to come back for repeat testing. Pre-clinical models of ACL injury have overcome these limitations by allowing for large sample sizes and longitudinal testing in controlled environments that permit repeat assessments of common measures. In addition, assessments of other important contributors to clinical outcomes (e.g., biomechanical variables) can be assessed concurrently with radiographic or histological measurements for subchondral bone or cartilage grade. Identifying changes in subchondral bone and understanding its relationship to articular cartilage can improve our understanding of pathogenic mechanisms of bone and cartilage

crosstalk following ACL injury and how they relate to commonly reported clinical outcomes (e.g., biomechanical variables).

2.4.4 Identifying Biochemical Cartilage Adaptations with Non-Invasive Imaging Modalities

Articular cartilage degeneration is the trademark of knee osteoarthritis, a whole-joint disease characterized by changes in cartilage morphology (volume and thickness) and composition (surface integrity and content).[144] Early identification of these cartilage adaptations has relied on non-invasive medical imaging modalities such as radiographs and magnetic resonance imaging (MRI) to detect changes in joint health. However, the utility of these imaging modalities to identify clinically meaningful adaptations in cartilage health is controversial. While radiographs are economical and quick to interpret, these images provide an indirect assessment of cartilage health by measuring joint space narrowing without the sensitivity to determine causation (i.e., whether narrowing occurred due to damage to surrounding joint structures or cartilage degeneration) or identify changes to intraarticular structures. Because of this pitfall, MRI has served as the gold standard of cartilage imaging, as it enables a comprehensive assessment of the whole knee joint including cartilage health with high sensitivity to detect pathology.[145-147] Furthermore, the molecular make-up of the compositional ECM (water, proteoglycan, collagen) leads to distinction between normal or degenerative cartilage that can be identified via MRI.[145-147]

In research settings, two primary quantitative cartilage mapping MRI sequences have gained traction: T_2 and $T_{1\rho}$. These sequences have been associated with the severity of knee osteoarthritis,[148,149] and are sensitive to different biochemical properties of the ECM including collagen orientation, and water and proteoglycan content. T_2 relaxation time is reflective of the free water flow in the cartilage ECM. Lower T_2 relaxation times are considered to be indicative of

healthy and hydrated cartilage with a strong collagen network, while increased T_2 relaxation times may reveal damage to the collagen fibrils and increase in water content.[119,150] In cases of idiopathic osteoarthritis, T_2 has correlated to disease severity characterized by the Kellgren-Lawrence grading system that classifies OA severity on radiographic images[151] and the semiquantitative whole-organ MRI scoring systems or WORMS.[152,153] Importantly, T_2 has been posited as a means to identify changes in water content and collagen prior to irreversible changes in articular cartilage, pointing to its ability to detect individuals early in the disease course.[119,152,154] Unlike T_2, $T_{1\rho}$ is reflective of the restriction of water inside the cartilage ECM.[119] It is collected through a spin-lock technique that reduces its dependence on dipolar interactions that are most prominent in the collagen ultrastructure of the ECM and therefore, is primarily reflective of proteoglycan content.[119,155] Increases in $T_{1\rho}$ relaxation time have been reported in individuals with idiopathic osteoarthritis with localized cartilage or subchondral bone lesions.[119,156,157] Similarly to T_2, changes in $T_{1\rho}$ relaxation has been shown to occur prior to irreversible structural changes.[158] Collectively, $T_{1\rho}$ and T_2 offer promising abilities to detect early biochemical changes at a time when the tissue may still be responsive to rehabilitation efforts.

It should be noted that conflicting findings of T_2 and $T_{1\rho}$ in patients with knee osteoarthritis have been reported in the literature,[156,159,160] which can complicate the clinical interpretation of these metrics.[161] In short, the dipolar interaction of collagen fibers in articular cartilage is minimized at certain angles (approximately 55 or 125°) between collagen fiber orientation and the main magnetic field of an MRI,[159] thereby increasing the relaxation times and can lead to conflicting results. The increase in relaxation times due to collagen orientation is known as the "magic angle effect."[159] Because $T_{1\rho}$ reduces dipolar interactions, T_2 is primarily affected by the magic angle.[119] Despite this, multiple studies have investigated $T_{1\rho}$ and T_2 following ACL injury

given their ability to detect biochemical changes before radiographic and clinical MRIs.[154,162] While increases in T_2 and $T_{1\rho}$ have been reported following ACL injury and ACLR,[157,163,164] the direct relationship to known and clinically affected modifiable risk factors (e.g., gait and muscle dysfunction) following ACL injury and ACLR remain largely unknown. Given that compositional changes in proteoglycan, water, and collagen content generally precede later staged structural abnormalities with PTOA, $T_{1\rho}$ and T_2 offers a promising ability to detect early changes in composition at a time when the tissue may still be responsive to rehabilitation efforts.

2.4.5 Biomechanical Risk Factors for PTOA after ACL Injury and Reconstruction

Following ACLR, altered sagittal plane knee mechanics are commonplace. In particular, patients with ACLR can have alterations in knee flexion angles and moments throughout stance, and particularly within the first year after surgery.[165,166] Despite being less studied than sagittal plane mechanics, adaptations in frontal plane mechanics have also been reported through alterations in the knee adduction moment, a surrogate measure of medial knee load.[165,167] Energy generation and absorption deficits are also apparent during walking and hopping tasks despite clearance to return to sport.[168,169] These biomechanical modifications in knee mechanics and energy transmission are thought to shift load distribution and potentially cause regions of cartilage that were previously adapted to load to become under- or over-loaded.[170,171] Given cartilage's viscoelastic and mechanical properties are responsive to load, this transition in load can alter tissue composition and induce degeneration.[171,172] While abnormal biomechanics have been consistently reported and have been theorized as significant risk factors that contribute to the PTOA development,[170,173-175] these data have primarily relied on cross-sectional study designs. Longitudinal data linking biomechanical adaptations to PTOA development would be powerful for evaluating disease progression and therapeutic windows of opportunity.

2.4.6 Utility of Experimental Animal Models for Assessments of PTOA

Preclinical models offer a unique and powerful approach to longitudinally examine the effect and interaction of different systems (e.g., muscular, inflammatory, neurological) involved in the pathogenesis of PTOA following ACL injury and ACLR. One of the main advantages of preclinical models is the ability to assess disease progression and identify modifiable targets without the introduction of confounding or limiting factors that commonly exist in human studies. For example, PTOA is often diagnosed when patients exhibit debilitating symptoms at the end stages of disease when the window for intervention has already passed. In addition, assessments in preclinical models can be fine-tuned to probe specific genes involved in the onset and progression of disease. As such, multiple preclinical models of ACL injury have been developed to study the etiological progression of PTOA. However, many existing models rely on invasive means to study the injury via the transection of the ACL that surgically violates the joint capsule to cut the ligament. Furthermore, many of the current models do not assess functional or clinical outcomes (e.g., biomechanics) in conjunction with the pathogenesis of PTOA. Evaluating gait kinematics (e.g., knee flexion angle) using technological advances and clinically translational models of ACL injury, may help elucidate the relationship between modifiable factors involved in joint loading and the development of PTOA.

2.4.7 Muscular Risk Factors for PTOA after ACL Injury and Reconstruction

One of the predominant theories of PTOA development following ACL injury is that neuromuscular impairments decrease the ability of the quadriceps to attenuate shock due to weakness, altering joint biomechanics and the distribution of load across articular cartilage.[170] The change in load alters the balance of turnover of the ECM components that comprise articular cartilage and consequently, initiates PTOA. While the association between quadriceps weakness

and idiopathic osteoarthritis has been well described,[176-180] data that links quadriceps weakness and PTOA development after ACL injury is sparse. Improving our understanding of the factors that influence quadriceps weakness, and furthermore, quadriceps dysfunction (e.g., changes in composition, architecture, and functional behavior), that contribute to PTOA development is critical to identify therapeutic targets for intervention.

The few studies that have investigated the relationship between quadriceps function and PTOA have used whole muscle metrics (e.g., isometric and isokinetic strength) and indirect assessments of cartilage health (e.g., radiography and clinical grading systems). Keays et al[181] showed isokinetic strength distinguished patients with early or moderate tibiofemoral PTOA measured by the Kellgren-Lawrence grading system that classifies OA severity on radiographic images.[151] Tourville et al[24] showed quadriceps strength at baseline prior to ACLR was associated with joint space width narrowing, also measured on radiographic images. Joint space narrowing is a measure that reflects structural deterioration of the joint and may precede the clinical diagnosis of PTOA. The work by Keays and Tourville et al[24,181] and others,[182] however, used only indirect measures of articular cartilage composition and structure (e.g., joint space width and clinical grading systems). Using advanced MRI techniques like $T_{1\rho}$ and T_2 can provide a more direct assessment of collagen orientation and proteoglycan content that may reveal changes in articular cartilage structure and composition that go undetected on radiographic images and improve the association between muscle strength and PTOA. Furthermore, as mentioned in the sections above, whole muscle strength does not provide insight to the intrinsic properties of muscle (e.g., composition, architecture, and functional behavior) that may better explain muscle dysfunction following ACL injury. As there is limited evidence to substantiate the association between muscle strength and PTOA, more comprehensive assessments of the intrinsic properties of muscle (e.g.,

composition, architecture, and functional behavior) may better explain the heightened risk of PTOA development following ACL injury. Future work would benefit from comprehensive assessments of quadriceps dysfunction and more direct assessments of articular cartilage structure and composition to determine if other factors of quadriceps's function are associated with articular cartilage degeneration. As such, this dissertation aims to uncover changes in muscle function following ACL injury that may help explain the heightened risk for developing PTOA through comprehensive assessments of muscle function (e.g., composition, architecture, and functional behavior) and more direct assessments of articular cartilage (e.g., $T_{1\rho}$ and T_2).

2.5   Conclusion

In summary, it is well known that ACL injury poses a significant hindrance to muscle strength recovery and knee function (e.g., biomechanical changes in gait) following ACL injury that increase the risk of developing PTOA. However, direct data linking knee function and muscle weakness to PTOA development remains largely unknown. To date, researchers have primarily relied on simplified measurements of muscle properties (e.g., size or volume) and graded scoring systems to diagnosis PTOA (e.g., joint space narrowing). However, these simplified measurements mask intrinsic muscle and cartilage properties that are important for joint function. As such, this dissertation aims to utilize the power of preclinical and human models and recent technological advancements to probe intrinsic properties of muscle and cartilage that have been largely understudied through 1) investigating the relationship between gait and early markers of PTOA (i.e., subchondral bone architecture) 2) comprehensively describing muscular function through assessments of both the quantity (e.g., volume) and quality (e.g., composition, architecture, functional behavior) of muscle and 3) improving our understanding of the relationship between muscle composition and PTOA following ACL injury. These studies offer unique contributions to

the literature by aiming to uncover properties that may help explain the high prevalence of muscle weakness and PTOA development following ACL injury and that also may be responsive to rehabilitation interventions for future work.

3   Joint Kinematics Dictate Subchondral Bone Remodeling in a Clinically Translational Model of ACL injury

This manuscript has been previously published in the Journal of Orthopedic Research.[183]

3.1   Abstract

Abnormal joint kinematics are commonly reported in the acute and chronic stages of recovery after anterior cruciate ligament (ACL) injury and have long been mechanistically implicated as a primary driver in the development of post-traumatic osteoarthritis. Though strongly theorized, it is unclear to what extent biomechanical adaptations after anterior cruciate ligament injury culminate in the development of post-traumatic osteoarthritis, as data that directly connects these factors does not exist. Using a pre-clinical, non-invasive ACL injury rodent model, our objective was to explore the direct effect of an isolated ACL injury on joint kinematics and the pathogenetic mechanisms involved in the development of post-traumatic osteoarthritis. Thirty-two, sixteen-week-old Long-Evans rats were exposed to a non-invasive ACL injury. Marker-less deep learning software (DeepLabCut) was used to track animal movement for sagittal-plane kinematic analyses and micro computed tomography was used to evaluate subchondral bone architecture at days 7, 14, 28 and 56 following injury. There was a significant decrease in peak knee flexion ($p<0.05$), which had a moderate-to-strong negative correlation ($r=-.59$ to $r=-.71$, $p<0.001$) with subchondral bone plate porosity in all load bearing regions of the femur and tibia. Additional comprehensive analyses of knee flexion profiles revealed dramatic alterations throughout the step cycle. This occurred alongside considerable

loss of epiphyseal trabecular bone and substantial changes in anatomical orientation. Knee flexion angle and subchondral bone microarchitecture are severely impacted after ACL injury. Reductions in peak knee flexion angle after ACL injury are directly associated with subchondral bone plate remodeling.

3.2   Introduction

Anterior cruciate ligament (ACL) injury is a debilitating condition that accelerates the onset of post-traumatic osteoarthritis (PTOA), a disease plaguing > 50% of ACL injured patients[184]. While most patients opt to receive surgical reconstruction, re-stabilizing the joint has been largely unsuccessful at reducing the risk of developing PTOA.[185] Abnormal joint kinetic and kinematics are commonly reported in the acute and chronic stages of recovery[173,186] and have long been mechanistically implicated as a primary driver of PTOA onset.[172] These biomechanical adaptations are thought to cause a non-uniform redistribution of mechanical load on articular cartilage,[172] which can lead to degenerative joint changes (identified by superficial fraying or splitting of cartilage and maladaptive subchondral bone architecture) associated with PTOA onset. Though strongly theorized,[172] it is unclear to what extent biomechanical adaptations after ACL injury culminate in the development of PTOA, as data that directly connects these factors does not exist. Work that can clearly establish this relationship represents a significant contribution to the literature, as it can be used to substantiate and develop empirically driven research programs.

The majority of work has used ACL transection models to study measures of PTOA.[135,187-193] Though practical, transection models lack in clinical translation as they violate the joint capsule creating a cascade of negative neuromuscular events that confound observations. For this reason, non-invasive models have been developed that more closely replicate the human mechanism of ACL injury and are particularly advantageous to fully characterize disease progression without the

confounding effects of surgical transection.[194-196] Despite this evolution, the concurrent investigation of joint kinematics with PTOA progression remains virtually unexplored,[197] even with it being a fundamental area of focus and rehabilitative tool for those with a history of joint injury.

To overcome many of these challenges that limit insight into disease progression, the goal of this study was to use our non-invasive rodent model of ACL injury to explore the direct effect of an isolated ACL injury on longitudinal joint kinematics and the pathogenetic mechanisms involved in the development of PTOA. To do this, we isolated observations directly to the injury by withholding all analgesics that are known to interfere with gait analysis[198] and bone remodeling.[199,200] Then, we used marker-less deep learning software, DeepLabCut,[201] to track animal movement and performed comprehensive kinematic analyses to better understand the longitudinal changes in knee kinematic profiles. We then explored subchondral bone architecture via micro computed tomography (μCT) to detect the association between hallmark measures of joint kinematics and PTOA after ACL injury. As a supplementary analysis, we also investigated sex and regional differences.

3.3 Methods

3.3.1 Experimental Animal Models

40 Long-Evans, 16-week-old male and female rats were obtained from Envigo, (Indianapolis, IN). All animals were housed in individual cages within the vivarium, had a one-week acclimatization period, and were allowed food and water *ad libitum* for the duration of the study. Prior to injury and during the one-week laboratory acclimatization period, all rats were also trained to walk on the motor-driven treadmill with a stable walking pattern. Animals were maintained in accordance with the National Institutes of Health guidelines on the care and use of

laboratory animals. This study was approved by our Institutional Animal Care and Use Committee (IACUC approval# A17-042).

3.3.2 Non-Invasive ACL Injury

A single load of tibial compression was used to induce knee injury to the right limb of all ACL injury rats. Briefly, rats were anesthetized (5% induction, 2% maintenance isoflurane/500 mL via a nose cone) and the ACL injury was induced in a custom built device (Figure 2) that was instrumented with a linear accelerator (8mm/s loading rate, model: DC linear actuator L16-63-12-P, Phidgets, Alberta, CA), load cell (HDM Inc., PW6D, Southfield, MI), and custom-written software program (LabVIEW, National Instruments, Austin, TX) that monitored the release of tibial compression during the load cycle, signifying an ACL tear. After the ACL rupture, a Lachman's test was performed to clinically confirm an ACL injury had occurred by detecting excessive anterior tibial translation while under the plane of anesthesia. The hindlimb was also palpated to detect any gross bone damage. If no contraindications were identified, the animal was transferred back to its cage and allowed to recover. ACL injuries were confirmed in all 32 injured rats upon dissection following μCT scanning (with no fractures, ruptures, or avulsions of other tissues). Mean compressive force at the knee injury for the females and males was 84.81 ± 11.9 N and 96.11 ± 28.2 N, respectively. Mean tearing rate of the knee injury for the females and males was 193.49 ± 32.3 N/s and 235.81 ± 51.3 N/s. Notably, in accordance with IACUC approval, analgesics were withheld throughout the duration of the study to ensure all findings were isolated to the injury, as the use of nonsteroidal anti-inflammatory drugs and opioids are known to directly interfere with the natural native biological response.[202,203]
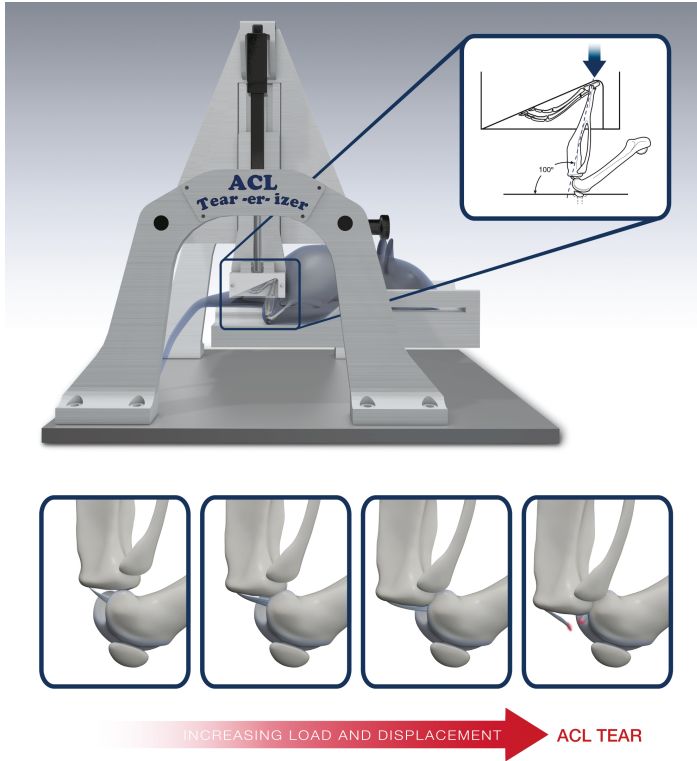
Figure 2: Mechanism of Injury. Custom built device used to tear a rat anterior cruciate ligament through a single load of tibial compression. The bottom plate holds the flexed knee, while the ankle is fixated into approximately 30° flexion. The knee is then loaded at a rate of 8mm/s, increasing the compressive forces on the tibia and causing anterior subluxation of the tibia relative to the femur. The compressive force imposed on the tibia releases when the anterior cruciate ligament is torn, signaling the device to retract.

3.3.3 Time points, gait collection, and analyses

To detail the time course of knee biomechanical adaptations after ACL injury associated with PTOA a longitudinal study design was utilized, where gait analyses were conducted at one of 4 time points (7, 14, 28, 56 days after injury). Before each session, the right hindlimb was shaved to better define the ankle, knee, and hip in each recording. Walking gait was then collected on a level-ground motor-driven treadmill (EXER 3/6 treadmill) set to 16 m/min pace and recorded at 250 frames per second using OptiTrack Prime Color cameras and Motive software (version 2.1.1).

DeepLabCut,[201] markerless pose-estimation software, was used to track the hip, knee, and ankle in the sagittal plane. Videos were clipped to 5-10 second durations. Frames were then

extracted and labeled by one single investigator (MSW) using DeepLabCut's graphical user interface. We used a ResNet-50 based neural network and trained it on 95% of the labeled images. A reiterative process was used to refine the model where frames with poor tracking results, through visual inspection or labels that jumped a pixel distance between frames, were extracted, relabeled, and added back to the model for retraining. The network was considered satisfactory when the training and test errors reached 2.7 and 5.39 pixels, respectively (660,000 iterations; image size: 1920x1080 pixels). We used a p-cutoff of 0.9 to condition the X,Y coordinates for future video analysis.

The trained network was applied to our unseen videos for analysis and used to predict kinematic landmarks of the hip, knee, and ankle joints (X,Y coordinates). The DeepLabCut-tracked trajectories were then imported into Python (3.7), where custom-written software was used to process all kinematic data. Knee sagittal plane angular displacement was extrapolated from the (X,Y) coordinates of each labeled landmark. Three consecutive step cycles for each rat were then extracted and each stride was time normalized to 100% of the step cycle that included both swing and stance phases. Average peak knee flexion angle was extracted from the stance phase of each rat from the time normalized strides.

3.3.4 Micro Computed Tomography Collection and Analyses

To quantitatively assess bone health associated with PTOA, the ACL injured rats were euthanized (via carbon dioxide overdose) following gait analysis at one of 4 time points (7, 14, 28, 56 days after injury). Uninjured control rats were euthanized on day 56. ACL-injured knee joints were scanned using μCT (Zeiss XRM Xradia 520 Versa, Zeiss Microscopy, Jena, Germany) with respective rodent bone settings (beam setting = 70kV/6W, pixel size = 12.25 μm) and subsequently

analyzed using Dragonfly image analysis software (version 4.1, Object Research Systems, Montreal, Quebec, Canada).

µCT data were imported in DICOM format, and a normalization filter was applied to uniformly distribute subject specific grayscale histograms. Image stacks were re-oriented such that two-dimensional view scenes were aligned with their respective anatomical views (axial, sagittal, and coronal). Images were then manually segmented by one blinded investigator (RJB) and then further subdivided into specific regions of interest (ROI's) (Figure 3).[194,195,204] Subchondral trabecular bone and the subchondral bone plate of the distal femur and proximal tibia were analyzed in the medial and lateral load bearing regions. Femoral load bearing regions were separated at the intercondylar notch and excluded the trochlear region, and tibial load bearing regions were separated at the intercondylar eminence.[194]

To quantify microarchitectural changes, multiple bone related measures of the subchondral trabecular and subchondral bone plate were calculated. Trabecular bone measures were sampled from the entire load bearing regions (Figure 3B, 3E) and included bone volume fraction (BV/TV, %), trabecular number (Tb.N, $1/mm$)[204] and tissue mineral density (TMD, mg HA/cm^3). Subchondral bone plate measures were sampled from a 1.04 x 1.04 mm^2 region of the subchondral bone plate centered in each the medial and lateral regions of the femur and tibia (Figure 3C, 3F)[205] and included subchondral bone plate thickness (SB.Pl.Th, µm) and porosity (Sb.Pl.Po, %). To determine TMD, grayscale units were transformed to hydroxyapatite concentration (mg HA/cm^3) using a bone phantom (model: Micro-CT HA D10, Quality Assurance in Radiology and Medicine, Moehrendorf, Germany) which was scanned with the same settings as the samples.

Clinically meaningful measures of PTOA were also assessed from µCT including the medial and lateral joint space width and anterior tibial translation. Joint space width was measured

as the minimum distance between the distal edge of the femoral condyle and the proximal edge of the tibial plateau. Anterior tibial translation was extracted from sagittal plane µCT images and measured as the perpendicular distance between a line tangent to the most posterior point of the femoral condyle to a line tangent to the most posterior point of the respective tibial plateau[206] on both the lateral and medial sides of the joint. To further extend insight, the femoral ACL enthesis was also explored using the aforementioned microarchitecture measures. This ROI was included, as emerging data indicate that this region of bone is particularly susceptible to PTOA, and thus is an important clinical consideration to more fully illustrate disease progression.[207,208] To capture this ROI, a 1.2 mm diameter cylinder was placed at a 55 degree angle[209] from the tibial articular surface in the frontal plane and at a 30 degree angle from the femoral articular surface in the axial plane such that the center of the cylinder intercepted the center of the enthesis (Figure 3G, 3G). Then, both trabecular and cortical bone were analyzed at the bottom third, or aperture of the cylindrical volume.[210] Supportive, qualitative images that characterize the extent of bone remodeling are depicted in Figure 4.
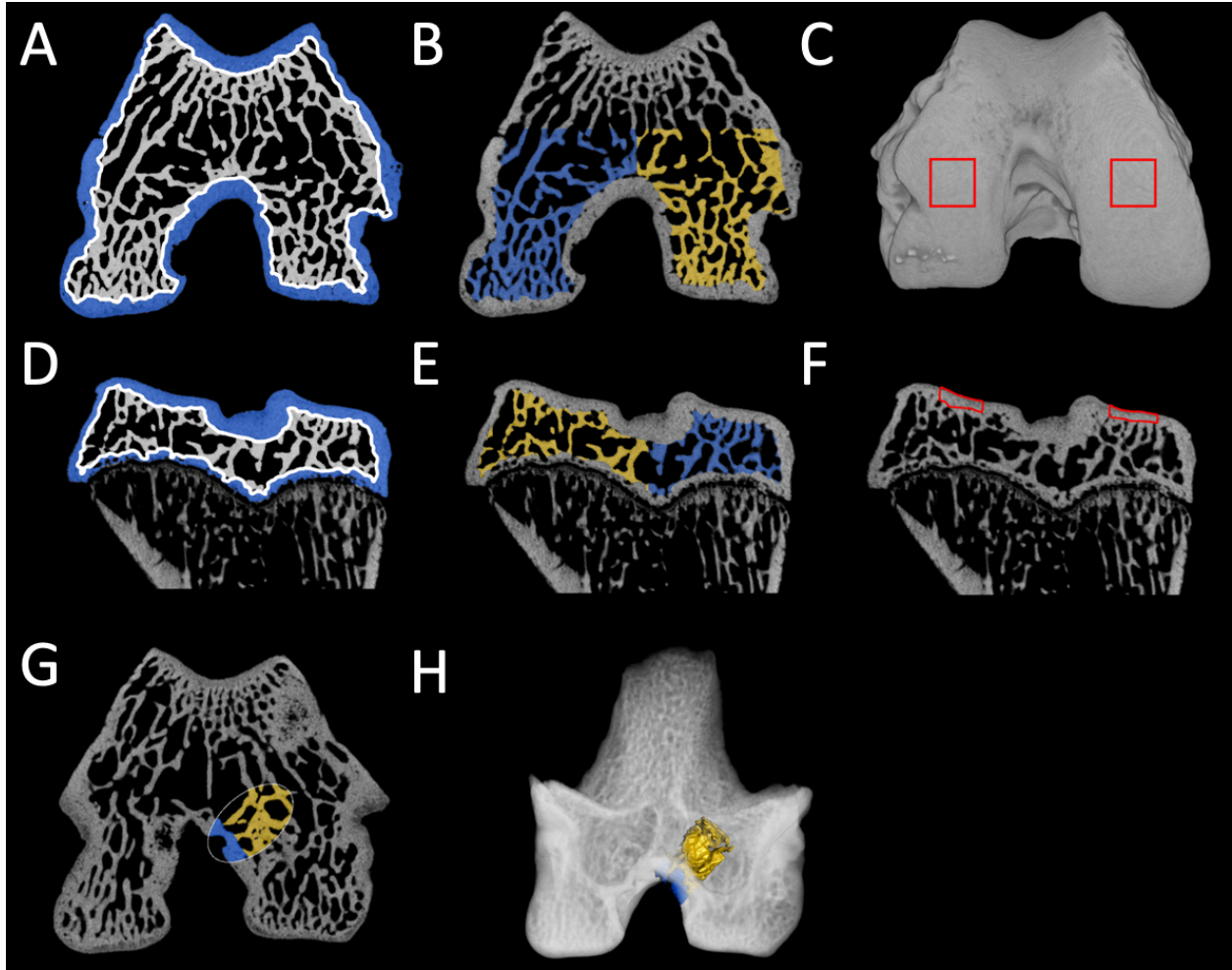
Figure 3: Micro computed tomography segmentations and regions of interest used in analyses. First, trabecular (white) and subchondral bone plate (blue) were segmented in the femur (A) and tibia (D). The segmentations were then subdivided into regions of interest for the medial (blue) and lateral (yellow) compartments for trabecular bone (B,E) and subchondral bone plate (C,F). The enthesis region of interest for trabecular bone (yellow) and subchondral bone plate (blue) are shown in (G) with a 3D representation in (H).

3.3.5   Statistical Analysis

To detail the time-course of knee biomechanical adaptions after ACL injury, functional analyses of variance were performed using the functional data analysis package in R (version 1.1.5). This approach treats knee joint angles as polynomial function thereby allowing for the detection of between-group (ACL-injured and control) differences at any percent of the step cycle rather than discrete time points.[211] Significant differences were considered to exist when 95%

confidence intervals did not overlap zero and were plotted on top of ensemble averages. To detail the time-course of PTOA after ACL injury, two-way analyses of variance (ANOVA) were performed on all μCT measures with factors of time since injury and sex (male/female) entered into the model. If there was no interaction effect, main effects of time since injury and/or sex were evaluated. In the case of a significant sex by time interaction post hoc one-way ANOVAs and independent t-tests were performed. Fisher's Least Significant Difference (LSD) post-hoc analyses were used to assess pair-wise effects for significant main effects relative to the Control group and interactions. To determine regional differences in the femur and the extent of anterior tibial translation, relative difference (relative difference $(x, x_{reference}) = \Delta \, / \, |x_{reference}|$) scores were calculated at each time point after ACL injury and two-way ANOVA were performed. For regional differences in the femur, factors of region (enthesis, lateral, medial) and sex were entered into the model. For anterior tibial translation, factors of time and sex were entered into the model with the post-hoc analysis as described above. Finally, to explore biomechanical factors that were associated with bony markers of PTOA after ACL injury, Pearson correlations were conducted to investigate the relationship between peak knee flexion angles during the stance phase of gait and porosity, an early-hallmark measure of PTOA[212] that is closely related to articular cartilage degeneration.[213,214] All analyses were performed using RStudio (version 1.1.5), with alpha levels of ≤ 0.05 required for statistical significance.
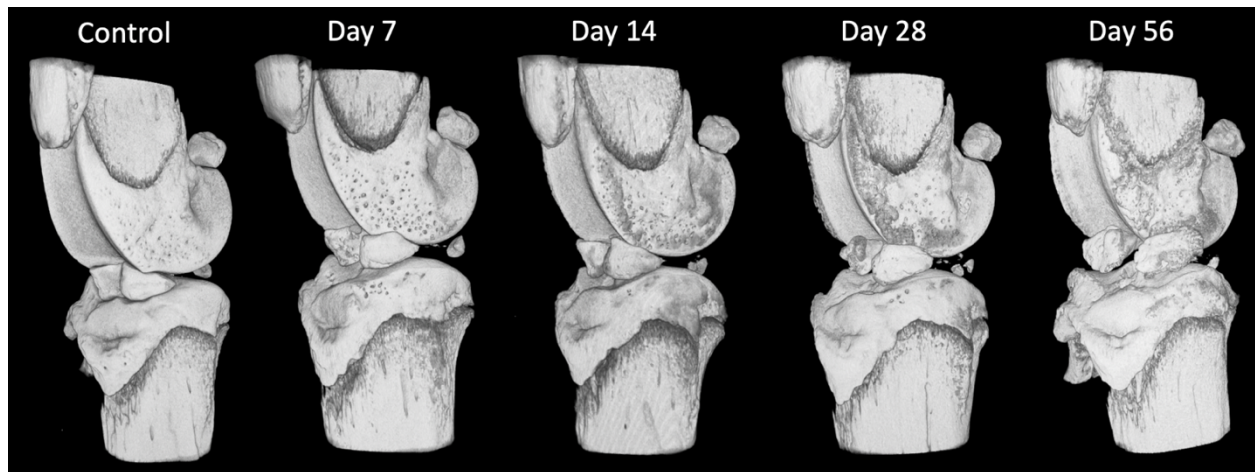
Figure 4: Representative bone structures by micro computed tomography at each time point following injury.

3.4 Results

The primary goal of our work was to directly explore the effect of an isolated ACL injury on longitudinal joint kinematics and the pathogenetic mechanisms of PTOA via detailed analyses of the subchondral bone. Hence, outcomes measures that directly illuminate this sequala and test this relationship are described in full detail below. Supportive outcomes measure that fully characterize our non-invasive ACL injury model are described in Tables 2 and 3, and Figures 6-9.

3.4.1 Knee Biomechanical Adaptations

Functional data analyses revealed significant differences between the Control group and ACL injury group at all time points following injury (Figure 5, $p<0.05$). At day 7 following injury, significant differences occurred during 51-72% (males) and 49-84% (females) of the step cycle and at day 14 following injury significant differences occurred during 28-40% (males) and 9-21% (females) of the step cycle. These changes equated to a 5-9% decrease in knee flexion angle compared to controls (Figure 5A-D, $p<0.05$). The most substantial alterations occurred at the later time points, day 28 and day 56 after injury (Figure 5E-H). At day 28 following injury, significant

differences occurred during 4-28% and 40-95% (males), and 1-16% and 46-88% (females) of the step cycle. At day 56 following injury significant differences occurred during 36-85% (males) and 1-5%, 31-78% (females) of the step cycle. During the later time points, rats walked with a 4-16% (males) or 6-14% (females) decrease in knee flexion angle compared to controls (Figure 5E-H, $p<0.05$). In addition, the changes during later time points occurred on average during 60% of the total step cycle compared to the earlier time point where rats walked significantly different than controls on average for only 20% of the total step cycle. Peak knee flexion angles were also significantly decreased at all time points ($p<0.001$) with no sex ($p=0.790$) or interaction effect ($p=0.132$).
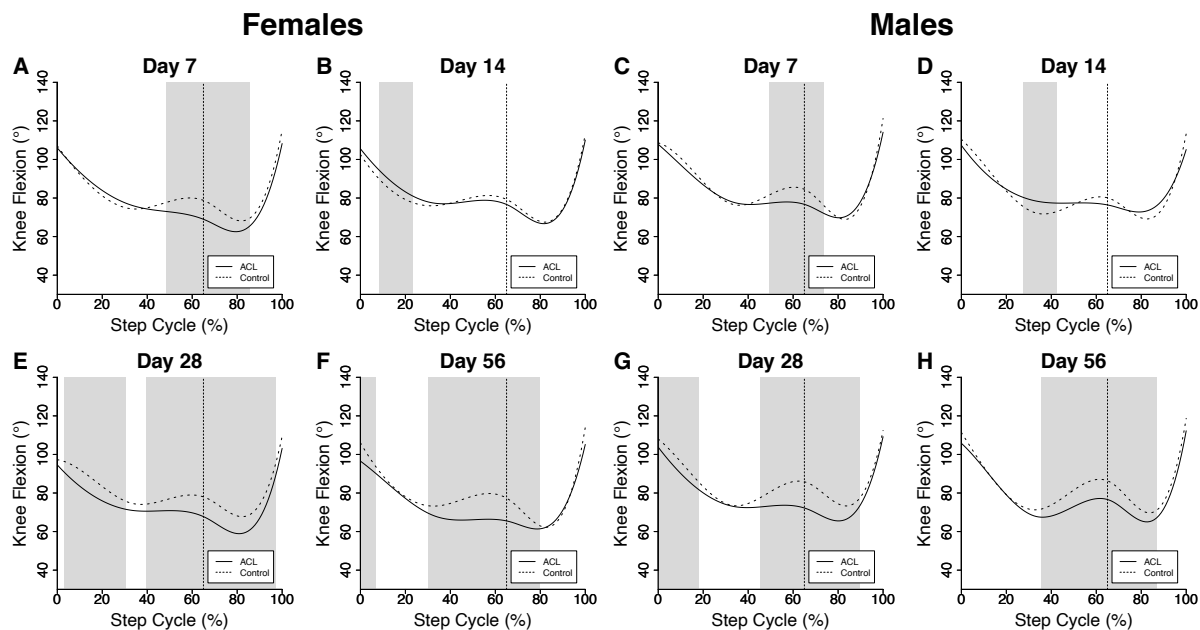
Figure 5: Longitudinal female (Panels A,B,E,F) and male (Panels C,D,G,H) kinematics and functional analyses of variances between anterior-cruciate-ligament-injured and control rats. The dashed vertical line indicates the percent of the step cycle where average peak knee flexion occurred. Average knee flexion angles across the step cycle are shown for each group with the shaded regions indicating significant differences between limbs.

3.4.2 Subchondral Bone Plate Remodeling

Subchondral bone plate remodeling was observed as a function of time and sex, with evidence in the medial and lateral femur and tibia (Table 1). In the lateral tibia, Sb.Pl.Th was significantly decreased at all time points following injury. In medial and lateral femur and tibia, a considerable loss of cortical bone was also observed where Sb.Pl.Po was found to be significantly elevated at every time point following injury. Notably, Sb.Pl.Po increased up to day 28 and was followed by a slight recovery at day 56 in all ROI's except the lateral femur where Sb.Pl.Po increased up to day 14 and had a slight recovery at days 28 and 56 but, none of the ROI's returned to a normative value.

3.4.3 Correlation analyses of knee biomechanical adaptations and PTOA markers

Significant moderate-to-strong negative correlations was observed between peak knee flexion angle and all Sb.Pl.Po outcomes in the medial (r=-.65, p<0.001) and lateral (r=-.71, p<0.001) femur (Figure 6), and medial (r=-.59, p<0.001) and lateral (r=-.64, p<0.001) tibia, indicating that reduced knee flexion angles were associated with greater Sb.Pl.Po, an early-hallmark bony measure of PTOA[212] that has been closely linked with articular cartilage degeneration.[213-215]

Knee kinematics correlate with porosity outcomes
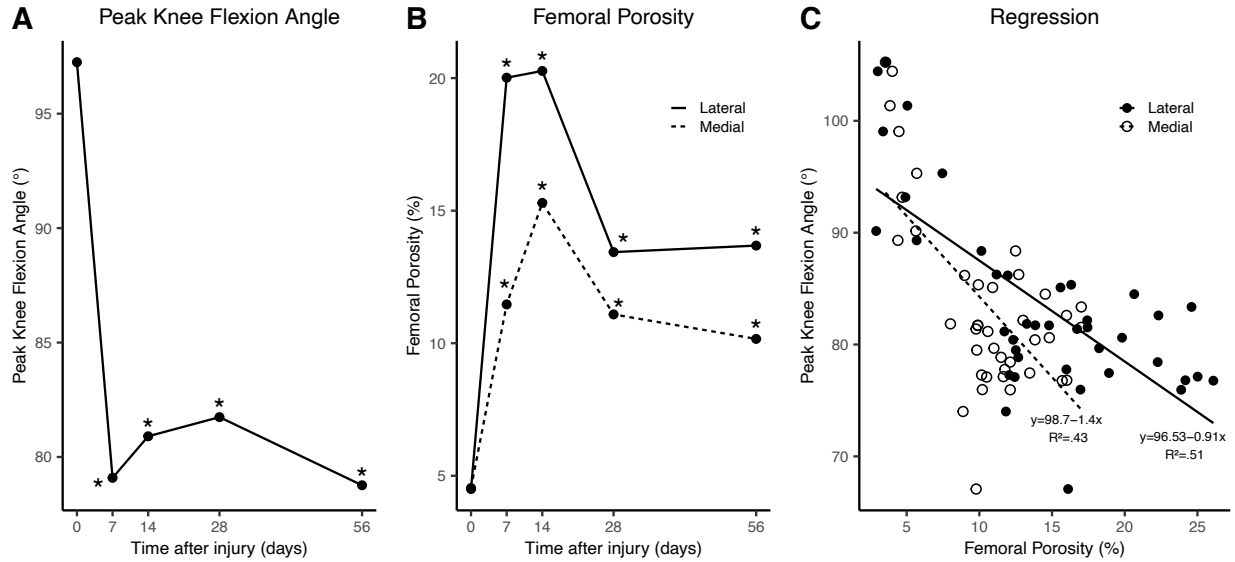
Figure 6: Representative correlational analyses between knee kinematics and femoral subchondral bone plate porosity. * indicates statistical significance (p<0.05) relative to the control group. A) Peak knee flexion angles were significantly decreased at all time points following injury (p<0.05) B) Porosity significantly increased at every time point after injury in the medial and lateral femur (p<0.05). C) Significant moderate-to-strong negative correlations were observed between peak knee flexion angle and porosity outcomes in the medial (r=-.65, p<0.001) and lateral femur (r=-.71, p<0.001) indicating that reduced knee flexion angles were associated with greater porosity, a hallmark bony indicator of post traumatic osteoarthritis. Notably, moderate negative correlations were also observed between peak knee flexion angle and porosity outcomes in the medial (r=-.59, p<0.001) and lateral (r=-.64, p<0.001) tibia (not shown in figure).

3.5 Discussion

We used our non-invasive, analgesic-free, animal model to investigate longitudinal knee kinematic function after ACL injury and its relationship to PTOA. We observed severe declines in joint kinematics through day 56 after injury, which were highlighted through discrete and functional data analyses of peak knee flexion angle (Figures 5, 6A). Significant subchondral bone plate remodeling was also observed, with a decrease in thickness (lateral tibia) and an increase in porosity in all load bearing regions at every time point post-injury (Table 1). Most notably, a direct link was made between reduced peak knee flexion angle and increased porosity (Figure 6). This occurred alongside the considerable loss of epiphyseal trabecular bone (Table 2) and substantial

changes in anatomical orientation (Table 3). Regional differences in the femur were also present between weight bearing ROI's and the enthesis, signifying a need for further investigation (Figures 7-10).

This is the first study to make a direct link between sagittal plane kinematics (clinical hallmark measures of gait) and PTOA in a non-invasive injury model. The observed smaller knee flexion angle at all time points was largely expected as this has been reported throughout the acute and chronic stages of recovery after ACL injury and reconstruction[173,186]. To extend this finding we incorporated functional data analyses, which is a novel application to the field as it has not yet been performed in rodent studies. The power of a functional data analysis is that it considers entire movement profiles and allows for the assessment of differences between limbs in both time and magnitude. With this analysis, we found the decline in knee kinematics to be immense, with significant changes exhibited throughout the entire step cycle and most predominantly at days 28 and 56. The observed decrease in knee flexion causes a redistribution of mechanical load and exacerbates joint instability and the mechanical stress induced from the time of injury. In our model, this coincided with the substantial increase in porosity. These findings align with previously reported data associating altered subchondral bone plate microstructure (porosity/perforations), articular cartilage degeneration, and mechanical loading. To this point, Botter et al[214] found an increased in porosity and significantly more cartilage damage 14 days after destabilization via intraarticular cartilage injection in a murine model. Iijima et al[213] further extended this idea by exploring the connection between subchondral bone plate perforations, articular cartilage degeneration, and mechanical loading after destabilization via medial meniscus surgery. Most notably, Iijima et al[213] found that subchondral bone plate perforations were localized to the load bearing region of the medial tibia which corresponded to localized regions of articular

cartilage degeneration. Our findings extend those of Botter and Iijima[213,214], by confirming a direct link through the moderate-to-strong correlations between peak knee flexion and subchondral bone plate remodeling in the load bearing ROI's (Figure 6).

As mentioned, there are differences in the injury mechanisms between Botter et al[214] (destabilization via intraarticular cartilage injection), Iijima et al[213] (destabilization via medial meniscus surgery) and the present study. The completely biological (without the use of injection or analgesics) and non-invasive mechanism of injury in our present study, isolates observations to the injury rather than introducing confounding factors with the use of non-biological agents or surgically induced models. However, regardless of the injury mechanism, all together our work and that of Botter[214] and Iijima[213] paint a compelling PTOA mechanism of action that can be attributed to the high levels of cross-talk between joint load (e.g. kinematics), subchondral bone remodeling and articular cartilage degeneration. Mechanistically, the microstructural changes to the subchondral bone can decrease interstitial pressure of articular cartilage and dampen its ability to adequately distribute load. [214,216] Microstructural changes can also facilitate a greater portal for biochemical interactions across the cartilage-bone interface, interfering with processes of maintaining bone health and potentially promoting PTOA.[213,214,216] Future work utilizing clinically translational models is needed to continue exploring these foundational relationships and mechanisms of PTOA action.

While not the primary goal of this study, we also characterized our model by investigating other bony microarchitecture metrics, anatomical orientations, and regional differences across the femoral ROI's after injury. Trabecular bone loss was considerable across time, sex, and region with the most observed loss occurring in the medial load bearing regions (Table 2). This agreed with the increase in lateral joint space width (Table 3), suggesting a shift toward medial loading

even though medial joint space width was unremarkable. In addition to lateral joint space width, there was substantial changes in medial and lateral anterior tibial translation, likely due to joint instability induced by the ACL injury (Table 3). Regional differences of the femur were also present across the medial, lateral, and enthesis ROI's (Figures 7-10). This observation is noteworthy and suggests a need to further investigate the ACL enthesis or bone-ligament interface as this area may be more susceptible to re-injury and therefore, may be a significant consideration in deciding optimal tunnel placement during reconstructive surgery and when to undergo surgical intervention.

Our study was not without limitations. First, we did not include contralateral limb comparisons, so it is possible that some of the observed changes in joint kinematics and bony morphology were present in both limbs. Second, we only included sagittal plane kinematics for this study. Extending future studies to cover three-dimensional joint kinematics may inform other modifiable factors beyond peak knee flexion angle that are linked to PTOA. Including analyses of articular cartilage can also help elucidate the crosstalk of the cartilage-bone interface and help identify which is the most prominent modifiable factor in PTOA that can be targeted through clinical intervention.

In conclusion, ACL injury dramatically alters joint kinematics and in turn, redistributes mechanical load throughout the joint. The altered kinematic patterns are associated with subchondral bone plate remodeling. This is the first study to make a direct link between sagittal plane kinematics and PTOA in a non-invasive clinically translational injury model.

3.6   Supplementary Files
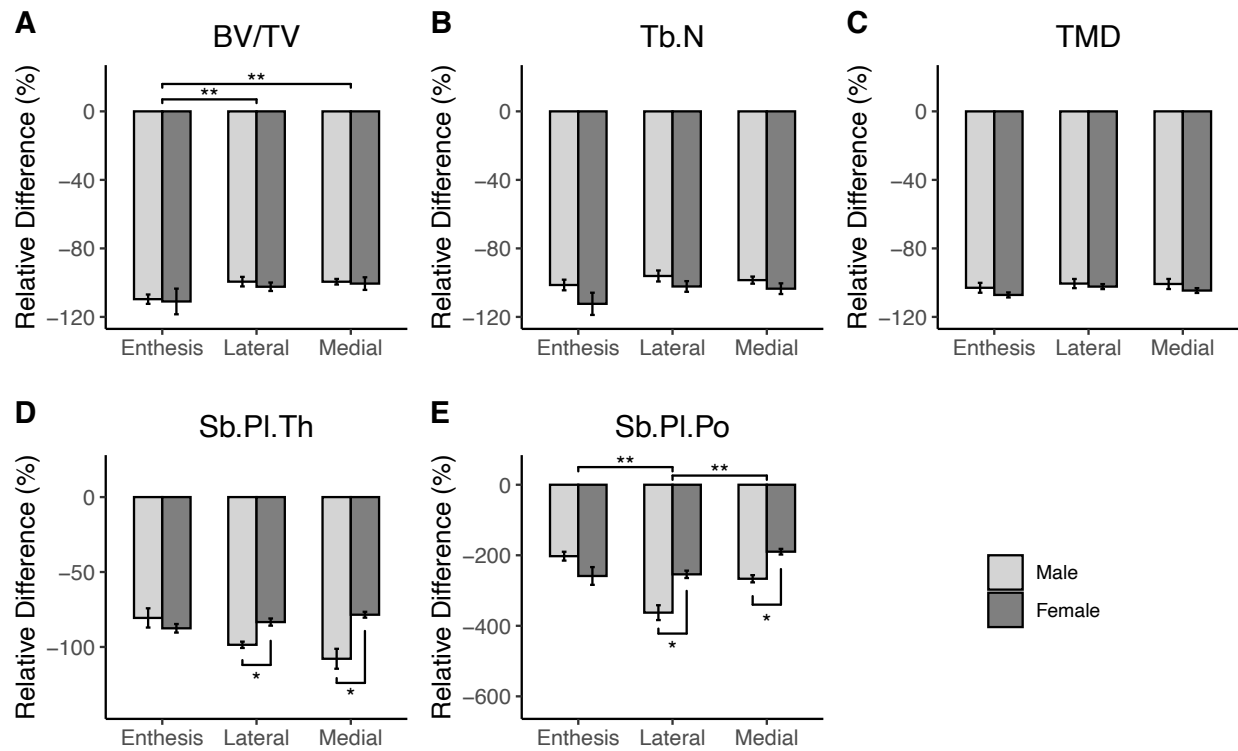
Figure 7: Regional differences in the femur at day 7 after injury. Data are represented as mean ± standard error. ** denotes significant differences between regions of interest, p<0.05. * denotes significant sex difference after injury (i.e. sex by time interaction), p<0.05. The enthesis was significantly more affected on BV/TV (A) and the lateral compartment was significantly more affected on Sb.Pl.Po compared to the medial compartment and enthesis region of interest. Significant differences between males and females occurred in the medial and lateral compartments on Sb.Pl.Th (D) and Sb.Pl.Po (E). Independent of injury, a significant main effect of sex was observed for Tb.N. Abbreviations: BV/TV, bone volume fraction; Tb.N, trabecular number; TMD, tissue mineral density; Sb.Pl.Th, subchondral bone plate thickness; Sb.Pl.Po, subchondral bone plate porosity.
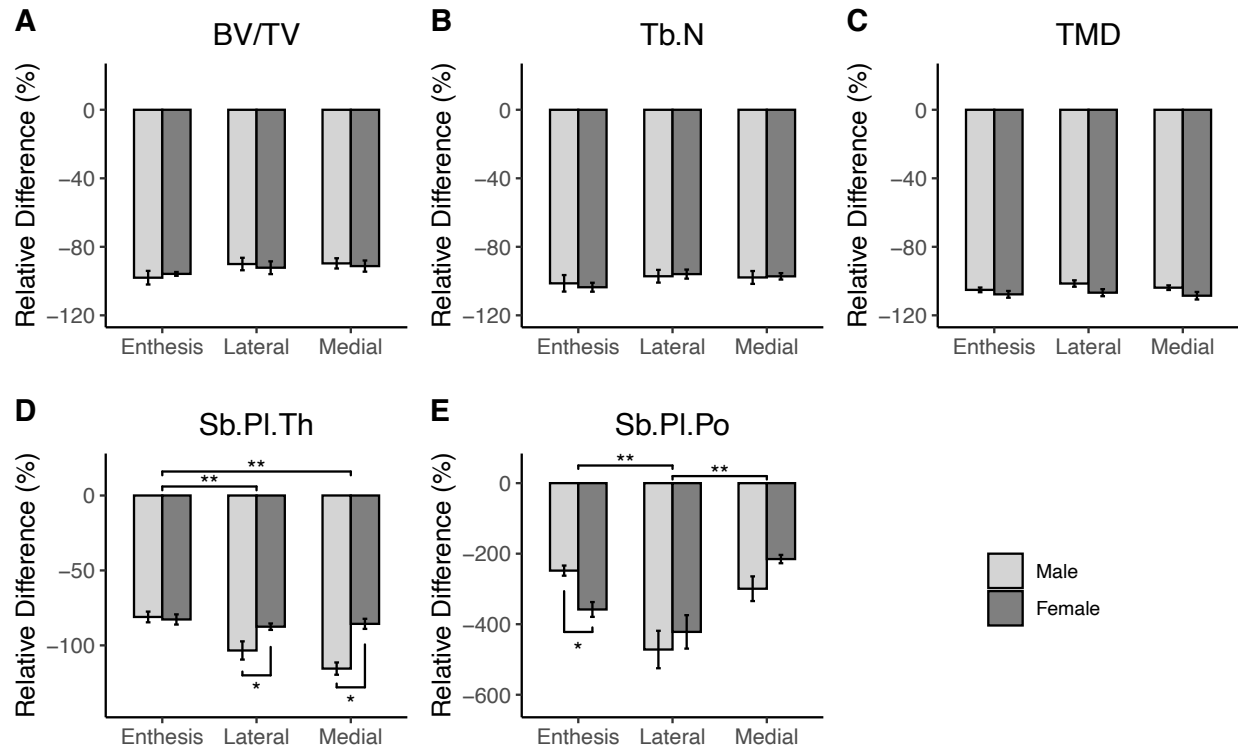
Figure 8: Regional differences in the femur at day 14 after injury. Data are represented as mean ± standard error. ** denotes significant differences between regions of interest, p<0.05. * denotes significant sex difference after injury (i.e. sex by time interaction), p<0.05. The enthesis region of interest was the least affected on Sb.Pl.Th, while the lateral compartment was affected the most on Sb.Pl.Po (p<0.05). (E). Significant differences between males and females occurred in the medial and lateral compartments on Sb.Pl.Th (D) and in the enthesis region of interest on Sb.Pl.Po (E). Independent of injury, a significant main effect of sex was also observed for TMD. Abbreviations: BV/TV, bone volume fraction; Tb.N, trabecular number; TMD, tissue mineral density; Sb.Pl.Th, subchondral bone plate thickness; Sb.Pl.Po, subchondral bone plate porosity.
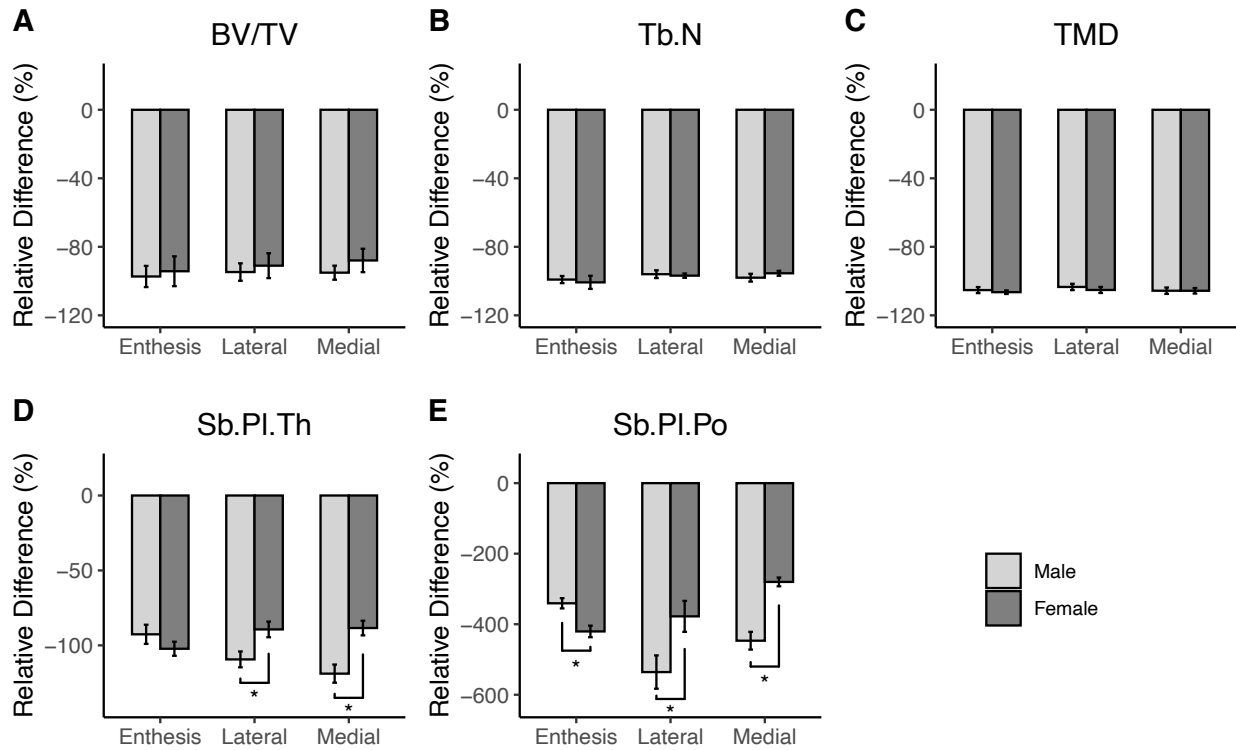
Figure 9: Regional differences in the femur at day 28 after injury. Data are represented as mean ± standard error. ** denotes significant differences between regions of interest, p<0.05. * denotes significant sex difference after injury (i.e. sex by time interaction), p<0.05. Significant differences between males and females occurred in the medial and lateral compartments on Sb.Pl.Th (D) and in all regions of interest on Sb.Pl.Po (E). Abbreviations: BV/TV, bone volume fraction; Tb.N, trabecular number; TMD, tissue mineral density; Sb.Pl.Th, subchondral bone plate thickness; Sb.Pl.Po, subchondral bone plate porosity.
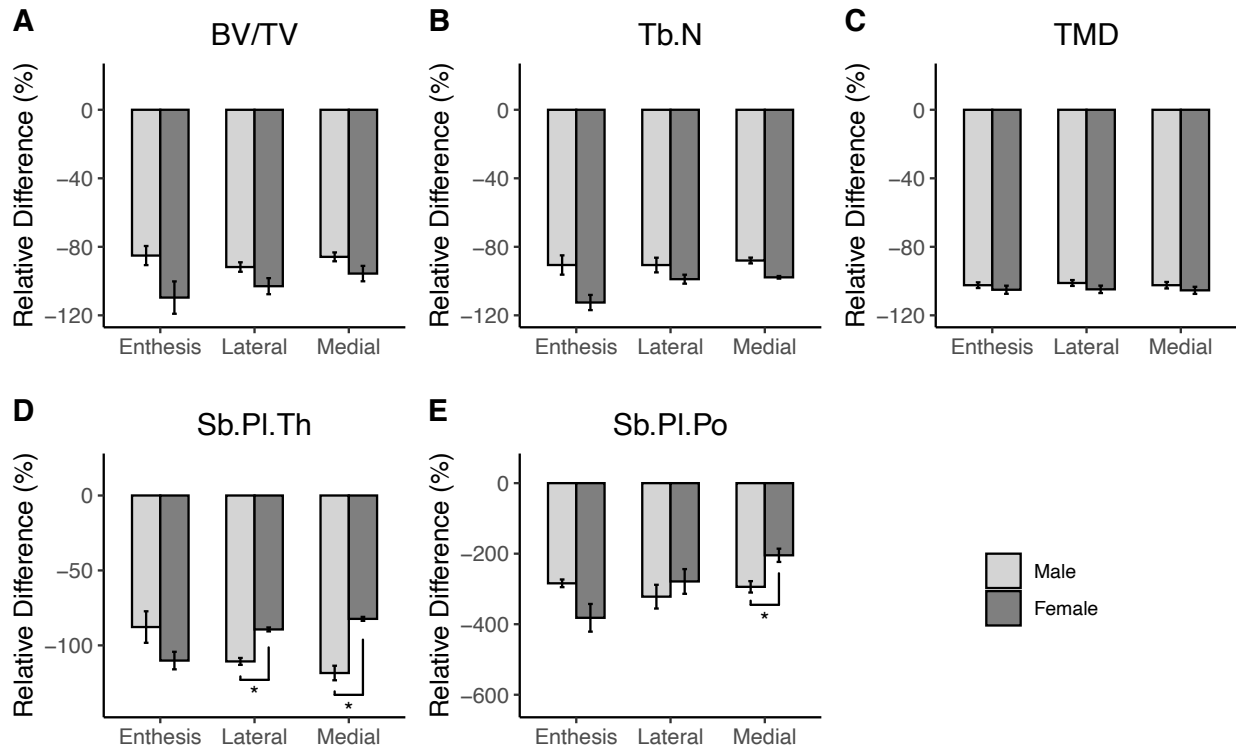
Figure 10: Regional differences in the femur at day 56 after injury. Data are represented as mean ± standard error. ** denotes significant differences between regions of interest, p<0.05. * denotes significant sex difference after injury (i.e. sex by time interaction), p < 0.05. Significant differences between males and females occurred in the medial and lateral compartments on Sb.Pl.Th (D) and in the medial compartment on Sb.Pl.Po (E). Independent of injury, significant main effects of sex were observed for BV/TV and Tb.N. Abbreviations: BV/TV, bone volume fraction; Tb.N, trabecular number; TMD, tissue mineral density; Sb.Pl.Th, subchondral bone plate thickness; Sb.Pl.Po, subchondral bone plate porosity.

<p style="text-align:center">Subchondral Bone Plate</p>

			Control	Day 7	Day 14	Day 28	Day 56
Femur							
Sb.Pl.Th (µm)	Lateral[α]	Females	202.86 ± 23.99	170.02 ± 6.67	164.46 ± 6.49	199.41 ± 13.01	184.55 ± 11.96
		Males	218.69 ± 8.51	206.22 ± 3.08	211.97 ± 21.02	198.70 ± 21.83	225.24 ± 8.67
	Medial[α]	Females	222.48 ± 17.67	170.60 ± 4.81	198.37 ± 5.82	200.36 ± 5.94	186.45 ± 4.43
		Males	233.12 ± 22.18	215.90 ± 12.64	231.65 ± 9.95	248.05 ± 10.29	241.83 ± 18.89
Sb.Pl.Po (%)	Lateral	Females	4.86 ± 1.06	12.34 ± 0.51^β	20.48 ± 2.30^β	18.34 ± 2.13^β	13.54 ± 1.70^β
		Males	4.14 ± 0.50	15.03 ± 0.87^β	19.55 ± 2.20^β	22.20 ± 1.96^β	13.34 ± 1.40^β
	Medial	Females	5.05 ± 0.36	9.59 ± 0.40^β	10.87 ± 0.60^β	14.53 ± 0.57^β	10.33 ± 0.95^β
		Males	4.03 ± 0.24	10.74 ± 0.41^β	12.06 ± 1.41^β	16.06 ± 0.59^β	11.84 ± 0.65^β
Tibia							
Sb.Pl.Th (µm)	Lateral[α]	Females	217.98 ± 13.43	176.49 ± 12.08^β	178.51 ± 5.45^β	168.66 ± 12.51^β	179.49 ± 11.47^β
		Males	257.59 ± 11.41	205.11 ± 11.02^β	220.84 ± 14.57^β	233.77 ± 22.69^β	231.47 ± 8.48^β
	Medial[α]	Females	218.52 ± 15.42	165.01 ± 8.77	155.67 ± 9.89	154.12 ± 10.16	170.21 ± 5.33
		Males	243.97 ± 12.68	236.16 ± 15.81	244.66 ± 21.17	289.60 ± 42.72	245.78 ± 17.15
Sb.Pl.Po (%)	Lateral	Females	7.93 ± 0.94	13.06 ± 0.99^β	17.72 ± 1.64^β	19.09 ± 1.92^β	13.90 ± 1.03^β
		Males	4.73 ± 0.93	11.73 ± 0.41^β	17.73 ± 2.60^β	19.21 ± 0.36^β	12.50 ± 0.43^β
	Medial	Females	6.20 ± 0.71	9.57 ± 0.42^β	11.71 ± 0.94^β	15.99 ± 1.59^β	11.62 ± 0.96^β
		Males	4.43 ± 0.63	10.30 ± 0.54^β	11.32 ± 0.55^β	18.03 ± 0.84^β	11.38 ± 1.06^β
Enthesis							
Sb.Pl.Th (µm)		Females	200.91 ± 8.45	175.89 ± 5.74^β	166.18 ± 6.80^β	205.45 ± 9.43	221.29 ± 11.75
		Males	232.41 ± 8.74	187.38 ± 14.92^β	188.36 ± 8.29^β	215.26 ± 14.85	204.00 ± 24.44
Sb.Pl.Po (%)		Females	4.26 ± 0.29	11.02 ± 1.07^β	15.26 ± 0.89^β	17.92 ± 0.70^β	16.27 ± 1.67^β
		Males	5.74 ± 0.22	11.63 ± 0.72^β	14.24 ± 0.83^β	19.55 ± 0.83^β	16.30 ± 0.63^β

Data are represented as mean ± standard error
Alpha level, p<0.05
[α]Significant main effect of sex regardless of time
[β]Significant main effect of time regardless of sex, relative to controls
Abbreviations: Sb.Pl.Th, subchondral bone plate thickness; Sb.Pl.Po, subchondral bone plate porosity

Table 1: Subchondral bone plate remodeling was observed as a function of time and sex, with evidence in the medial and lateral femur and tibia. Most notably, Sb.Pl.Po was found to be significantly elevated at every time point following injury in all regions of interest (p < 0.05).

			Epiphyseal Trabecular Bone				
			Control	Day 7	Day 14	Day 28	Day 56
Femur							
BV/TV (%)	Lateral	Females	0.42 ± 0.01	0.43 ± 0.01	0.39 ± 0.02	0.38 ± 0.03	0.43 ± 0.02
		Males	0.43 ± 0.01	0.42 ± 0.01	0.39 ± 0.02	0.41 ± 0.02	0.39 ± 0.01
	Medial	Females	0.46 ± 0.01	0.46 ± 0.02	0.42 ± 0.01[β]	0.40 ± 0.03[β]	0.44 ± 0.02[β]
		Males	0.46 ± 0.01	0.46 ± 0.01	0.41 ± 0.01[β]	0.44 ± 0.02[β]	0.40 ± 0.01[β]
Tb.N (1/mm)	Lateral[α]	Females	3.91 ± 0.05	3.99 ± 0.12	3.75 ± 0.10	3.78 ± 0.05	3.86 ± 0.10
		Males	3.78 ± 0.04	3.63 ± 0.12	3.67 ± 0.14	3.63 ± 0.09	3.42 ± 0.16
	Medial[α][γ]	Females	4.08 ± 0.03[δ]	4.22 ± 0.13[δ]	3.97 ± 0.08	3.89 ± 0.06	3.99 ± 0.03[β][δ]
		Males	3.84 ± 0.05[γ]	3.78 ± 0.08[γ]	3.75 ± 0.14[γ]	3.76 ± 0.09[γ]	3.38 ± 0.06[β]
TMD (mg HA/cm³)	Lateral	Females	1039.41 ± 41.59	1063.30 ± 15.15	1109.95 ± 21.40	1093.43 ± 18.24	1089.62 ± 22.47
		Males	1087.65 ± 31.36	1093.39 ± 29.07	1103.33 ± 20.27	1125.05 ± 20.10	1099.93 ± 18.59
	Medial	Females	1031.83 ± 36.93	1079.38 ± 14.49	1120.12 ± 22.80	1090.29 ± 16.59	1087.75 ± 21.34
		Males	1080.76 ± 29.06	1089.16 ± 31.76	1122.46 ± 14.54	1141.70 ± 20.12	1106.87 ± 20.54
Tibia							
BV/TV (%)	Lateral	Females	0.40 ± 0.01	0.41 ± 0.01	0.38 ± 0.02	0.36 ± 0.03	0.38 ± 0.02
		Males	0.39 ± 0.01	0.39 ± 0.01	0.34 ± 0.01	0.37 ± 0.02	0.35 ± 0.01
	Medial[α]	Females	0.46 ± 0.01	0.45 ± 0.01	0.44 ± 0.02[β]	0.41 ± 0.04[β]	0.40 ± 0.02[β]
		Males	0.45 ± 0.00	0.42 ± 0.01	0.38 ± 0.01[β]	0.39 ± 0.01[β]	0.35 ± 0.02[β]
Tb.N (1/mm)	Lateral[α]	Females	4.11 ± 0.07	4.16 ± 0.10	3.88 ± 0.12[β]	3.79 ± 0.10[β]	3.96 ± 0.08[β]
		Males	3.72 ± 0.08	3.62 ± 0.13	3.43 ± 0.10[β]	3.52 ± 0.10[β]	3.41 ± 0.12[β]
	Medial[α]	Females	4.08 ± 0.08	4.09 ± 0.13	3.91 ± 0.08[β]	3.87 ± 0.07[β]	3.91 ± 0.08[β]
		Males	3.72 ± 0.06	3.63 ± 0.08	3.43 ± 0.12[β]	3.36 ± 0.07[β]	3.14 ± 0.19[β]
TMD (mg HA/cm³)	Lateral[α]	Females	996.49 ± 35.03	1020.11 ± 20.46	987.54 ± 102.47	927.00 ± 99.80	1067.14 ± 17.20
		Males	1052.31 ± 31.06	1057.27 ± 32.58	1081.38 ± 21.05	1101.41 ± 16.89	1066.18 ± 19.63
	Medial	Females	1031.30 ± 35.24	1066.01 ± 21.01	1021.94 ± 103.14	1057.26 ± 45.35	1092.85 ± 22.20
		Males	1080.63 ± 32.73	1088.26 ± 35.68	1108.91 ± 18.92	1128.40 ± 19.39	1095.75 ± 16.90
Enthesis							
BV/TV[α] (%)		Females	0.35 ± 0.00	0.39 ± 0.03	0.34 ± 0.00	0.33 ± 0.03	0.39 ± 0.03
		Males	0.41 ± 0.01	0.45 ± 0.01	0.40 ± 0.02	0.40 ± 0.03	0.35 ± 0.02
Tb.N (1/mm)		Females	3.25 ± 0.05	3.65 ± 0.21	3.36 ± 0.08	3.27 ± 0.12	3.65 ± 0.14
		Males	3.36 ± 0.13	3.41 ± 0.10	3.40 ± 0.16	3.33 ± 0.07	3.05 ± 0.19
TMD (mg HA/cm³)		Females	1022.90 ± 39.08	1096.31 ± 14.98	1102.19 ± 20.18	1089.33 ± 10.68	1074.81 ± 24.21
		Males	1067.36 ± 29.33	1099.33 ± 30.87	1122.35 ± 14.57	1123.61 ± 18.81	1093.12 ± 17.76

Data are represented as mean ± standard error
Alpha level, $p<0.05$
[α]Significant main effect of sex regardless of time
[β]Significant main effect of time regardless of sex, relative to controls
[γ]Significant interaction effect. Post hoc analyses revealed different from Day 56
[δ]Significant interaction effect. Post hoc analyses revealed different from males
Abbreviations: BV/TV, bone volume fraction; Tb.N, trabecular number; TMD, tissue mineral density

Table 2: Femoral and tibial trabecular bone remodeling was observed as a function of time and sex, and significant changes primarily resided in the medial compartments starting 14 days after injury. Notably, BV/TV was significantly decreased in the medial femoral and tibial compartments and Tb.N was significantly decreased in the medial tibial compartment. In the lateral tibia, Tb.N showed a significant decrease starting at day 14 after injury. All significant sex effects are denoted in the table. No significant main time or sex effects, or interaction effects were found on BV/TV, Tb.N, or TMD in the enthesis region of interest ($p > 0.05$).

		Control[ε]	Day 7	Day 14	Day 28	Day 56
				Anatomical Orientation		
ATT (%)						
Lateral[α]	Females		$-7.58 \pm 47^{\delta}$	$-17.39 \pm 32^{\delta}$	$-5.64 \pm 34^{\delta}$	45.93 ± 40
	Males		$-8.00 \pm 8^{\delta}$	$46.29 \pm 9^{\delta}$	$65.93 \pm 18^{\delta}$	136.65 ± 16
Medial	Females		$13.51 \pm 5^{\delta}$	$42.84 \pm 46^{\delta}$	$16.84 \pm 30^{\delta}$	119.72 ± 18
	Males		$-4.73 \pm 11^{\delta}$	$7.15 \pm 24^{\delta}$	$42.60 \pm 17^{\delta}$	98.17 ± 13
JSW (µm)						
Lateral[α]	Females	126.62 ± 21	$278.32 \pm 113^{\beta}$	$266.55 \pm 77^{\beta}$	$315.35 \pm 119^{\beta}$	$244.88 \pm 77^{\beta}$
	Males	86.28 ± 19	$418.51 \pm 70^{\beta}$	$554.70 \pm 36^{\beta}$	$440.23 \pm 94^{\beta}$	$541.40 \pm 151^{\beta}$
Medial	Females	236.21 ± 38	367.83 ± 46	389.93 ± 104	295.11 ± 74	401.35 ± 38
	Males	227.51 ± 32	326.61 ± 50	345.15 ± 17	305.93 ± 58	382.69 ± 147

Data are represented as mean ± standard error

[ε]ATT not reported because used to compute relative difference scores

Alpha level, $p<0.05$

[α]Significant main effect of sex regardless of time

[β]Significant main effect of time regardless of sex, relative to controls

[δ]Significant main effect of time regardless of sex. Post hoc analyses revealed different from Day 56

Abbreviations: ATT, anterior tibial translation; JSW, joint space width

Table 3: Medial and lateral anterior tibial translation significantly increased over time. Anterior-cruciate-ligament-injured males and females on average increased 98% and 120% respectively on the medial side and 137% and 46% respectively on the lateral side of the knee joint. Lateral joint space width was also found to be significantly increased at every time point following injury. All significant sex effects are denoted in the table. No significant changes in joint space width were indicated on the medial side (p > 0.05).

4   The Role of Intramuscular Fat on In-Vivo Fascicle Mechanics During Walking Following Anterior Cruciate Ligament Reconstruction

4.1   Abstract

Recent work has highlighted that muscle weakness following anterior cruciate ligament reconstruction (ACLR) is part of a broader profile of muscle dysfunction, characterized by intrinsic changes of muscle properties such as tissue quality and mechanical behavior. These properties are vital for generating force and facilitate shape changes during movement. Elevated accumulation of fibro-adipogenic progenitors and expansions of the extracellular matrix have been observed post-ACLR, which may lead to the accumulation of non-contractile elements like intramuscular fat and potentially affecting fascicle mechanics. Therefore, our main objectives were to I) utilize *in-vivo* B-mode ultrasound imaging to comprehensively evaluate if vastus lateralis fascicle mechanics are altered in individuals with a protracted history of ACLR during walking at a self-selected pace and II) to understand their relationship with intramuscular fat and knee mechanics. Twenty-four individuals with primary ACLR and 24 healthy controls underwent two experimental testing sessions to assess 1) quadriceps strength, and fascicle and knee mechanics and 2) muscle volume and intramuscular fat of the vastus lateralis via quantitative magnetic resonance imaging. Linear mixed effects models were used to assess (I) quadriceps strength, (II) fascicle mechanics including fascicle length and angle excursion, and fascicle length and angle at heel strike and peak knee extension moment (III) intramuscular fat and fat-cleared muscle volume, (IV) knee mechanics including peak knee extension moment (KEM), instantaneous rate of moment

development (RMD). Regression analyses were run to better understand the relationships between intramuscular fat and fascicle mechanics, as well as between fascicle and knee mechanics. We demonstrate that patients with a history of chronic ACLR exhibit between-limb strength and fascicle excursions (length and angle) that are not significantly different than those of healthy controls. As it relates to knee mechanics, we observed a smaller fascicle angle at peak KEM in the ACLR-involved limb (p < 0.017), which was associated with a decrease in joint load measured by peak KEM and RMD (p = 0.020-0.031). This indicates an abnormality in fascicle architecture specifically during the peak demand of quadriceps force production. Though, the ACLR limb exhibited significant atrophy (p < 0.001), we did not find any differences in intramuscular fat relative to healthy controls or any associations between intramuscular fat and fascicle outcomes (p > 0.05). These data indicate that after ACLR, a muscle's overall change in shape (fascicle length and angle) is not different than healthy controls, however, there are fascicle angle deficits that occur when greatest demand is placed on the quadriceps muscle that cannot be attributed to muscle atrophy or intramuscular fat.

4.2 Introduction

One of the primary targets of post-operative rehabilitation following anterior cruciate ligament reconstruction (ACLR) is quadriceps weakness given its association with increased reinjury risk and a host of compensatory biomechanical strategies that are adopted after ACLR.[217] Despite targeted rehabilitation efforts, only 20% of patients are able to regain sufficient strength in their ACL limb at the time of return to sport.[1] Furthermore, even in the chronic stages of recovery (>12 months), these patients continue to exhibit alterations in knee mechanics, such as reduced knee flexion excursions (KFE) and knee extensor moments (KEM)[218-220]. Quadriceps weakness has been primarily attributed to neurological impairments (i.e., impaired cortico-spinal

drive, spinal-reflexive alterations) and whole muscle atrophy.[6-8] While these factors are important, neurological impairments and whole muscle atrophy do not capture the intrinsic properties of muscle that ultimately govern its ability to generate force and withstand load. Recent studies have started to acknowledge that muscle weakness indeed falls under a larger multifaceted profile of global muscle dysfunction, observed by changes in intrinsic properties of muscle such as tissue quality (i.e., composition) and a muscle's mechanical behavior (i.e., muscle mechanics).[29,62,63] However, there is a notable lack of research on tissue quality and muscle mechanics following ACLR, leading to fundamental gaps in our understanding of their prevalence and functional impact in this patient population.

A crucial determinant of quadriceps force production is a muscle's architecture, such as its fascicle length and angle. Fascicle length is an estimate of muscle fiber length, while fascicle angle refers to the angle at which muscle fascicles are oriented to the muscle's line of action. These architectural properties, both individually and in combination, have an impact on how muscle architecture adapts and changes shape during movement and subsequently, affects the muscle's force-length and force-velocity properties. Due to recent technological advancements in medical ultrasound imaging, these two key components of muscle architecture can be non-invasively assessed *in vivo* during both static and dynamic conditions (e.g., isometric strength, or during walking). The ability to assess muscle architecture *in vivo* has provided important insight into how muscles differentially function during activities of daily living. For example, although the predominant thought is that the quadriceps act eccentrically during walking, recent *in vivo* studies have been able to detect that the vastus lateralis (VL) actually contracts isometrically or even concentrically, and allows most force transmission to occur through tendon lengthening rather than fascicle lengthening in healthy limbs.[46] In contrast to this, in the acute stages following ACLR (7.5

± 1.2 months), *in vivo* ultrasound has shown fascicles undergo eccentric lengthening in the ACLR limb during weight acceptance.[63] While these findings are interesting, it remains unknown if the observed differences in fascicle mechanics persist in a more chronic cohort who have long since completed rehabilitation and returned to activities of daily living. By examining fascicle mechanics during walking, it also becomes feasible to explore the relationship between fascicle and knee mechanics. Considering the occurrence of abnormal knee mechanics following ACLR (such as changes in KEM,[221,222] joint power,[63] and rates of torque and moment development[223,224]), it is also crucial to ascertain whether the associations between fascicle and knee mechanics persist in chronic populations. This comprehension could potentially offer valuable insights for intervention strategies.

A factor that may influence fascicle mechanics in chronic cohorts is the accumulation of non-contractile elements, such as intramuscular fat. An increase in intramuscular fat can impair a muscle's mechanical properties.[225] For example, modeling studies have shown that higher levels of fat can elevate muscle stiffness and modify muscle quality and in turn, influence the interaction between muscle fibers and aponeuroses. Consequently, the altered interaction between muscle fibers and aponeuroses can result in reduced force production by the muscle.[225] This phenomenon has been observed in populations known to exhibit quadriceps strength deficits, such as inactive individuals,[226] obese individuals,[227] the elderly,[95] and those with knee osteoarthritis.[98] Following ACL injury, elevated levels of fibrogenic/adipogenic progenitors (FAPs) have been found in the ACL-injured limb,[37,40] which can disrupt extracellular matrix remodeling, inhibit satellite cell formation, contribute to high levels of inflammation, and promote the differentiation of fat and fibrotic tissue[40,78,228]. Others have also shown an increased frequency of quadriceps hypoechoic signaling, a preliminary indicator of fat accumulation measured via B-mode ultrasound, following

ACLR.[99] As such, it remains plausible that non-contractile elements like intramuscular fat accumulate following ACLR and contribute to persistent quadriceps dysfunction following ACLR. Importantly, an imaging modality like magnetic resonance imaging (MRI), which possesses a higher degree of sensitivity than B-mode ultrasound imaging to quantify whole muscle intramuscular fat, has not yet been performed following ACLR but can serve as a comprehensive assessment to determine the prevalence of intramuscular fat following ACLR.

As persistent muscle weakness and biomechanical adaptations hinder full recovery following ACLR and can contribute to joint degeneration, it is plausible that fascicle mechanics may be altered in the presence of intramuscular fat and contribute to a range of poor biomechanical outcomes commonly observed after ACLR. Therefore, our main objective was to comprehensively evaluate if fascicle mechanics are altered in individuals with a protracted history of ACLR during walking. We hypothesized that those with a chronic history of ACLR would exhibit reduced fascicle length and angle excursion (primary outcomes) compared to healthy controls. A secondary objective was to determine if alterations in fascicle mechanics could be explained by compositional changes induced by the buildup of intramuscular fat (i.e., higher fat fraction). We hypothesized that those with a chronic history of ACLR would have an increase in intramuscular fat (secondary outcome) compared to healthy controls, and that this would be associated with reduced fascicle length and angle excursions. Lastly, we explored relationships between fascicle and knee specific biomechanical outcomes known to be problematic following ACLR, herein referred to as knee mechanics, such as peak KEM, instantaneous rate of moment development (RMD), peak power absorption, and KFE, to investigate whether fascicle-level adaptions might influence boarder aspects of knee-related physical function.

4.3 Methods

4.3.1 Experimental Design

Twenty-four individuals who had undergone primary unilateral ACLR were recruited from the Department of Orthopaedic Surgery and the general student population at the University of Michigan (Age: 22.8 ± 3.6 years, Time since surgery: 3.3 ± 0.09 years). ACLR individuals were eligible for this study if they: 1) were between 14-45 years of age 2) had no prior knee injury or surgery other than the current ACL 3) were between 1.5-5 years post-ACLR 4) had received a bone patellar tendon bone graft and 5) had a body mass index (BMI) under 28 kg/m^2. ACLR participants were excluded if they 1) had multiple ACLR's unilaterally or bilaterally, and/or 2) have any graft type other than a bone patella tendon bone. To characterize healthy profiles, a convenience cohort of twenty-four healthy individuals were also recruited (Age: 22.0 ± 3.1 years) that had no history of lower extremity injury or surgery and had a BMI under 28 kg/m^2. Demographic information for included participants can be found in Table 4.

All participants meeting the above criteria for either group underwent two experimental sessions over two days. The first experimental session consisted of patient reported outcomes, strength testing, and fascicle mechanics measured synchronously during self-selected gait on an instrumented treadmill, where limb order was randomized. The second experimental session consisted of MRI to measure intramuscular fat via mDixon MRI sequence. All sessions were completed on average 12 ± 14 days of one another. All participants provided informed consent, and all protocols were approved by the University of Michigan Medical School Institutional Review Board (IRBMED: HUM00169174).

	ACLR (n = 24)	Control (n = 24)	p value
Females/Males	15/9	14/10	-
Age (yrs)	22.8 ± 3.6	22.0 ± 3.1	0.406
Height (m)	1.70 ± 0.1	1.70 ± 0.1	0.856
Weight (kg)	68.7 ± 8.9	69.9 ± 13.6	0.732
Body Mass Index	23.2 ± 1.9	23.3 ± 2.6	0.801
Time after surgery (yr)	3.3 ± 0.9	N/A	-
Tegner Activity Scale	Pre: 8.4 ± 1.1 Post: 6.8 ± 1.6	Pre: 6.8 ± 1.3 Post: 6.7 ± 1.2	Pre: <0.001* Post: 0.815
KOOS Symptoms	84.7 ± 10.6	95.7 ± 5.4	<0.001*
KOOS Pain	95.4 ± 5.3	98.7 ± 2.3	0.007
KOOS Activities of Daily Living	99.0 ± 1.4	99.8 ± 0.9	0.039
KOOS Sports and Recreation	89.0 ± 8.5	98.3 ± 4.3	<0.001*
KOOS Quality of Life	83.1 ± 14.6	99.2 ± 2.1	<0.001*
IKDC	88.9 ± 8.8	98.7 ± 3.0	<0.001*

Table 4. Subject Demographic and Patient Reported Outcomes. Data are reported as mean ± standard deviation.*Denotes statistical significance at the level of $p<0.05$. Abbreviations: KOOS = Knee Injury and Osteoarthritis Outcome Score; IKDC = International Knee Documentation Committee

4.3.2 Participant Reported Outcomes

Participants completed a series of questionnaires to evaluate self-reported function including the Tegner Physical Activity,[229] International Knee Documentation Committee (IKDC)[230] and Knee Injury and Osteoarthritis Outcome Score (KOOS).[231]

4.3.3 Isometric Quadriceps Strength

Isometric knee extensor strength was assessed as previously described using an isokinetic dynamometer (CSMi Humac Norms Stoughton, MA).[232] Participants were provided strong verbal encouragement and real-time visual feedback to encourage patients to exert maximal effort during all testing contractions.

The knee joint was positioned at 60° of flexion.[233] Participants then completed a standardized warm-up protocol consisting of two practice repetitions at each of the following intensities: 25%, 50%, and 75% of perceived maximal effort. Following these warm-up contractions, participants completed one practice trial at 100% of their perceived maximal effort. The maximal voluntary knee extensor torque during this final practice trial was then used to set a visual torque target for the subsequent MVC assessments. For test trials, all participants performed three MVC's in which they were instructed to kick out as hard and fast as they can. Participants were given two minutes of rest between each trial. Peak knee extension (KE) torque (N·m) was extracted from each MVC trial. Peak torque was normalized to body mass (N·m/kg) and the peak of peaks was used in the subsequent analyses.
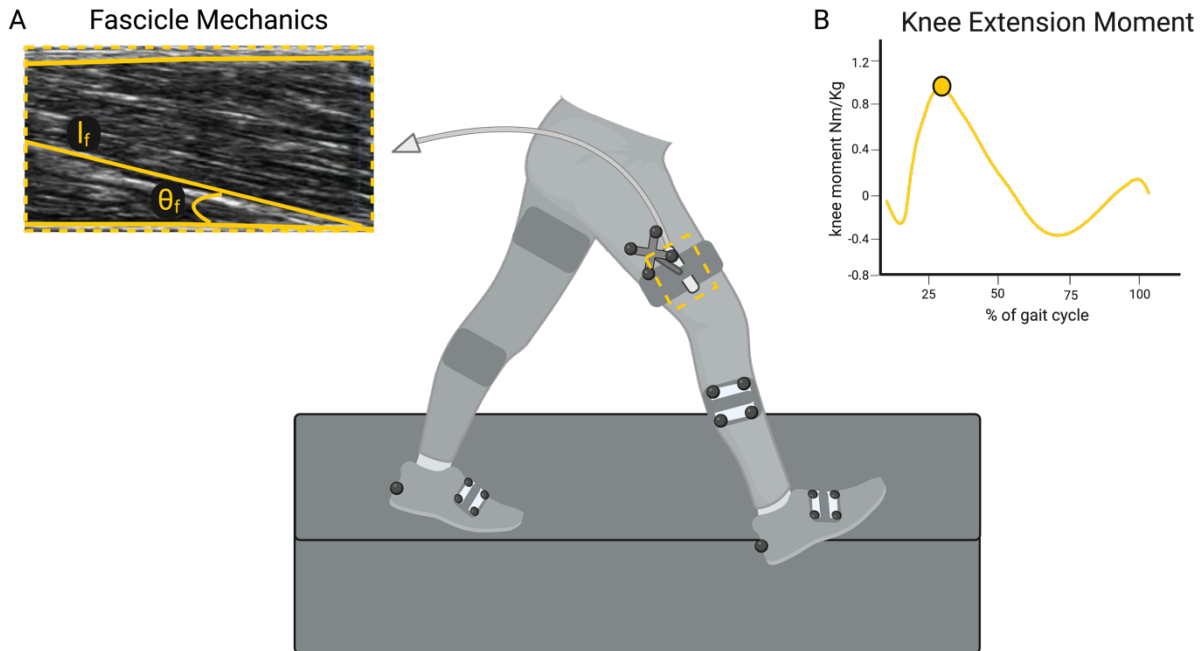
Figure 11: Simultaneous Assessments of Fascicle and Knee Mechanics with Representative Dynamic Markers. In vivo (A) fascicle length (lf) and angle (θf) were recorded at 100 frames/second and extracted at every frame from heel strike to peak knee extension moment (B). Representative forelimb clusters (middle) show custom marker cluster on thigh that attached to the ultrasound mount. Created with BioRender.com

4.3.4   Assessments of Knee and Fascicle Mechanics

Self-selected over-ground walking speed was determined as an average of five trials on a 20-m walkway using infrared timing gates (model TF100, TrakTronix, Lenexa KS) and was used to set the treadmill speed (m/s) for all subsequent biomechanical and ultrasonic assessments.[234,235] A total of 48 retroreflective markers were used to construct our biomechanical model. Static markers were placed bilaterally on the iliac crests, anterior superior iliac spine, greater trochanter, medial and lateral femoral epicondyles, medial and lateral malleoli, and the first and fifth metatarsal heads. Dynamic markers were placed bilaterally on the calcaneus and rigid clusters of four non-collinear markers affixed on the sacrum, bilaterally on the thigh, shank, and foot

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segments.[236] An initial standing calibration trial was captured, and static markers were subsequently removed leaving only the rigid clusters and calcaneus markers.[236]

In order to record B-mode images of longitudinal cross-sections of the VL, a single 60 mm ultrasound transducer (LV8-5N60-A2; ArtUS, Telemed, Vilnius, Lithuania) was affixed midway between the greater trochanter and lateral femoral condyle[237] using a ProbeFix Dynamic T custom mount (Usono, Eindhoven, The Netherlands) and elastic wraps (Figure 11) on one limb at a time. To ensure consistency in placement, the transducer was affixed to the limb being tested by a single trained laboratory member. To ensure optimal transducer orientation, the participant flexed and extended their knee while standing and as needed, micro-adjustments were made to limit out of plane motion.

Lower extremity biomechanics were collected at the self-selected walking pace on a split-belt instrumented treadmill (Bertec, Columbus, Ohio) recording ground reaction force data at 2000 Hz and synchronized with a 10-camera motion capture system (Qualisys, Gothenburg, Sweden) recording at 200 Hz. Patients acclimated to the dual-belt, instrumented treadmill for 5 minutes. Then, three 12 s trials of simultaneous gait, force plate, and ultrasound data were collected. B-mode ultrasound videos of the VL were captured at 100 frames/s at a depth of 50 mm. Motion capture and ground reaction force data were synced with ultrasound by a 5V trigger signal to indicate the start and end of the ultrasound recordings. The above process was repeated to capture a new static trial and subsequent biomechanical and ultrasonic assessments on the opposite limb.

Motion capture and force data were exported to Visual 3D (C-Motion Inc., Germantown MD) and the kinematic model was created. A low-pass Butterworth filter with cutoff frequencies of 6 and 12 Hz were applied to the motion capture and force data, respectively. Hip joint center was estimated using the Davis method,[238] while knee and ankle joint centers were estimated using

half the distance between the medial and lateral epicondyles and malleoli, respectively. Knee angles were calculated using the Cardan rotation sequence (x= flexion/extension, y=ab/adduction, z=internal/external rotation) and were negated for descriptive purposes such that positive angles represented knee flexion and negative angles represented knee extension. Filtered kinematic and kinetic data were then submitted to a standard inverse dynamic's procedure within Visual 3D and joint moments were reported as internal. A 20N threshold was used to determine heel strike[239] Stance was defined by heel strike and toe-off.

For subsequent assessments of the relationship between fascicle and knee mechanics, and to ensure our ACLR cohort was representative of established trends for knee mechanics following ACLR, time-normalized profiles were calculated and averaged across strides for knee angle, moments, and powers.[221] Joint moments were normalized by body mass (kg) and height (m). Peak KEM was extracted from the first 50% of stance. Peak power absorption (i.e., negative joint power), average instantaneous RMD, and KFE were extracted between heel strike and peak KEM. All outcome variables reported are from the limb the ultrasound transducers were placed on to ensure consistency in our comparisons between limbs and to reduce errors introduced by ultrasound placements on the limb.[63]

B-mode ultrasound videos of the VL were processed using a combination of open-sourced and custom-written software. First, all ultrasound videos recorded during the motion capture trials were clipped to only include data during the stance phase of gait. The 12 s motion capture trials resulted in participants taking 6-9 complete step cycles. Next, all videos were processed through an open sourced program DL Track (https://github.com/njcronin/DL_Track/tree/master).[240] Using a combination of pre-segmented training data provided from DL Track and additional frames of manually-segmented VL frames from the current study data, a single neural network was trained

using DL Track in order to extract position data of all detected (via neural network) fascicles and aponeuroses on each frame.[240] Fascicle lengths and angles were extracted from DL Track. Fascicle lengths and angles exceeding 3 standard deviations from the mean per frame were excluded to reduce variability caused by inaccurate neural network tracking. To further improve confidence in our outcomes, all filtered position data from DL track were then imported into a custom software application written in MATLAB (version 2021a, The MathWorks, Natick, USA) to perform manual adjustments of positional data as needed and as determined by a single trained lab personnel. A test-retest analysis of our custom-built application was performed on fascicle length and angle excursions by 1 rater on n = 4 limbs with excellent reliability (ranging 0.944-0.969; Supplementary Table 13). The same test-retest analysis was performed on discrete outcomes including fascicle length and angle at heel-strike and peak KEM with moderate to good reliability (ranging 0.510- 0.848; Supplementary Table 13).

Fascicle length was measured from the deep to superficial aponeuroses. In frames where fascicles extended beyond the image, the intersection of the fascicle and superficial aponeuroses was estimated by a linear projection of the fascicle.[241] Fascicle angle was measured as the angle of the fascicle relative to the deep aponeuroses. On average, three to five fascicles were tracked on each frame from heel strike to peak KEM. The mean fascicle length and angle per frame were submitted to subsequent analyses. Fascicle length and angle trajectories were filtered using a window average, normalized to 100%, and averaged across three step cycles per limb. Fascicle length and angle were extracted at discrete points in stance (heel strike and peak KEM), while fascicle length and angle excursion were measured as the total movement from heel strike to peak KEM considering the cumulative change in position between every frame. A cumulative excursion value in the negative range indicates that fascicle mechanics can be described as a shortening event

and/or there was a reduction in fascicle angles from the initial heel strike to the instant of peak KEM.

4.3.5 Quantification of Intramuscular Fat

MRI data of the bilateral upper thighs were acquired with a 3-Tesla MRI Philips Ingenia scanner using a 16-element anterior torso coil and 12-element receive coil located within the table coil. Three stacks were acquired with a 30-mm overlap covering a field of view (FOV) of 420 x 284 x 140 mm^3. Stack-specific shimming was applied independently to each stack to minimize B0 field inhomogeneity. An axial mDixon Quant sequence was performed with the following key acquisition parameters: 3D gradient echo; number of echoes = 6 (1st echo time = 1.2 ms, change in echo time = 0.9 ms); repetition time = 7.283 ms; flip angle = 3°; acquired matrix size = 264 x 178 mm; reconstructed matrix size = 288 x 288 mm; acquired voxel size = 1.6 x 1.6 x 2.2 mm^3; reconstructed voxel size = 1.3 x 1.3 x 1.1 mm^3; parallel imaging factor SENSE = 2; number of slices/stack = 740.

In post-processing, five slices on the proximal and distal ends of each stack were removed to reduce signal inhomogeneity that occurs at the ends of each stack. In the regions of overlap, slices closest to the center of the stack were retained. Stacks were then stitched together to generate fat only, water only, in phase, out of phase, and fat fraction images. To reduce the number of slices and maintain the signal-to-noise ratio from the acquisition, signals were averaged over every four slices. A limited number of slices of bilateral VL's were segmented on the water-only image and a semi-automatic method using a combination of diffeomorphic registrations was used to propagate these segmentations to the remaining slices.[242,243] Propagations were refined in ITK snap.[244] Custom-written software was used to erode a single voxel border from all muscle segmentations before extracting median fat fractions to protect against manual error in

segmentations that may have included subcutaneous fat and therefore, confound our measurement of intramuscular fat. Muscle volume was extracted from the full region of interest. While controlling for sex, subject mass and height were significantly correlated to each other ($r = 0.799$) and therefore, muscle volume was normalized to subject mass only. Fat-cleared muscle volume was computed by subtracting the median fat fraction from the normalized muscle volume.[245]

4.3.6 Statistical Analyses

An a priori power calculation using data from previously published work[62] that investigated fascicle mechanics (fascicle length and angle excursion) during strength testing was completed to estimate sample size. The power calculation for a two factor analysis of variance (ANOVA) revealed that a minimum of 11 individuals with a history of primary ACLR and 11 healthy controls were needed to detect a significant difference between groups and limbs (alpha = 0.05 and power = 0.8) (G*Power v3.1.9.6).[246]

Statistical analyses were conducted using R Statistical Software (4.2.2, Vienna, Austria) with a significance level set at $p \leq 0.05$. Participant demographics and patient reported outcomes between the ACLR and Control groups were compared using independent t-tests (Table 4). To account for between-limb variability in the Control subjects, both Control limbs were included in subsequent linear mixed effect model analyses. Given that almost all of our subjects considered their right-leg as dominant (n=47 of 48) and 9 of 24 ACLR subjects received ACLR on their left limb, we randomly chose 9 Control subjects and assigned their left limb as involved, while the other Control subjects had their right limb assigned to involved. The limb that received surgery in the ACLR group was assigned to the ACLR-involved limb, while the contralateral limb was assigned to ACLR-uninvolved.

The following variables were analyzed using linear mixed effects models (lmer function from the lmerTest and lme4 packages in R [247,248]): (I) Quadriceps strength, (II) fascicle mechanics including fascicle length and angle excursion, and fascicle length and angle at heel strike and peak KEM (III) intramuscular fat and fat-cleared muscle volume, (IV) knee mechanics including peak KEM, RMD, peak power absorption, and KFE. The fixed factors in the models included limb (involved vs. uninvolved), group (ACLR vs. Control), and their interaction. Subject was treated as a random factor and sex and BMI were included as control variables. The interaction term compared the differences between limbs (injured-uninjured) for each group. In the case of a significant group by limb interaction, post hoc analyses were conducted using the emmeans package[249] to calculate that estimated marginal means for each group and perform pairwise comparisons between groups with Tukey adjustment for multiple comparisons.

Regression analyses were run to understand the relationships between intramuscular fat and fascicle mechanics, as well as between fascicle and knee mechanics. Notably, these multiple regression analyses were only run with variables and limbs that were deemed to be statistically different from the aforementioned linear mixed effect models. As such, the regression analyses only included the ACLR-involved, ACLR-uninvolved, and Control-involved limbs as no differences were detected between the Control-involved and Control-uninvolved for the selected variables. Interaction terms were included in the model to determine if limb influenced the relationship between fat and fascicle mechanics, and fascicle and knee mechanics. Subject was included as a random factor in all models. In the case of a significant interaction, simple slope analyses were performed using the sim_slopes function from the jtools package in R.[250] To assess the explained variance, separate regressions were also performed for each limb.

4.4 Results

4.4.1 Participant Reported Outcomes and Quadriceps Strength

KOOS subscale and IKDC results are summarized in Table 4 and quadriceps strength is summarized in Table 5. The ACLR cohort has significantly worse outcomes on all KOOS subscales and IKDC knee-specific outcomes than the control cohort (p = <0.001 – 0.039). There was a significant group by limb interaction for quadriceps strength. However, post-hoc analyses indicated the differences were small and non-significant (p = 0.098-0.249).

	ACLR Involved	ACLR Uninvolved	Control Involved	Control Uninvolved	t	p value
Peak Torque (N·m/kg)	2.96 ± 0.51	3.12 ± 0.45	3.13 ± 0.77	3.01 ± 0.68	-2.02	0.049*

Table 5. Data are reported as mean ± standard deviation. t statistic and p value reported is result of the limb by group interaction from the linear mixed effects model, with * denoting a statistically interaction at the level of p<0.05. However, post-hoc analyses did not reveal any between-limb differences within each group (p < 0.05).

4.4.2 Fascicle Mechanics

Fascicles mechanics are summarized in Table 6 and Figures 12 and 13. No significant interactions or main effects were noted for fascicle length or angle excursion (p > 0.05; Figure 12C/13C). When investigating fascicle length at the discrete points in stance (HS and peak KEM) there were no significant interactions between group and limb, however there was a main effect of limb that revealed the involved limbs had longer fascicles at peak KEM relative to the uninvolved limbs, independent of group (p = 0.014; Figure 12A/B). When investigating fascicle angle at discrete points in stance (HS and peak KEM), there was a significant group by limb interaction at peak KEM (p = 0.017; Figure 13B) but not at HS (p = 0.078; Figure 13A). Post hoc analyses

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indicated that the ACLR-involved limb exhibited a fascicle angle of 2.14 degrees less than the ACLR-uninvolved limb at peak KEM (p <0.001; Figure 13B). No difference between limbs was noted for the Control group (p = 0.336). Additionally, there was a main effect for limb at HS (t[46] = 2.84, p = 0.007), indicating that the uninvolved limbs had an increased fascicle angle, independent of group (Figure 13A). Overall, these findings indicate fascicles angles in the ACLR-involved limb are smaller at peak KEM but fascicle excursion throughout the duration of the movement do not differ.

	Measure	ACLR Involved	ACLR Uninvolved	Control Involved	Control Uninvolved	t	p value
Fascicle Length (cm)	Heel Strike	11.32 ± 2.98	10.52 ± 3.35	11.46 ± 3.02	11.19 ± 3.07	0.57	0.569
	Peak KEM$^{\alpha}$	10.99 ± 2.46	10.00 ± 2.66	10.85 ± 2.57	10.58 ± 2.37	1.30	0.201
	Excursion	-0.54 ± 1.65	-0.28 ± 0.59	-0.33 ± 1.86	-0.58 ± 1.02	-0.93	0.355
Fascicle Angle (°)	Heel Strike$^{\beta}$	10.72 ± 2.12	12.01 ± 2.46	11.18 ± 2.45	11.32 ± 2.22	-1.80	0.078
	Peak KEM	**10.59 ± 2.38**	**12.73 ± 2.56**	11.69 ± 2.05	12.16 ± 2.09	-2.49	0.017*
	Excursion	0.10 ± 0.90	0.49 ± 0.84	0.42 ± 0.93	0.69 ± 0.80	-0.35	0.730

Table 6. Fascicle Excursions During Weight Acceptance and Results of Interaction from Linear Mixed Effects Model. Data are reported as mean ± standard deviation. t statistics and p values reported are results of the limb by group interaction from the linear mixed effects model, with * denoting a statistically interaction at the level of p < 0.05. Bold text indicates that post-hoc analyses revealed that the significant limb by group interaction stemmed from between-limb differences within the group (p < 0.05). $^{\alpha}$ or $^{\beta}$ denote a significant main effect of limb, where the involved limbs had longer fascicle lengths and smaller fascicle angles relative to the uninvolved limbs, respectively. Abbreviations: KEM = Knee Extensor Moment.
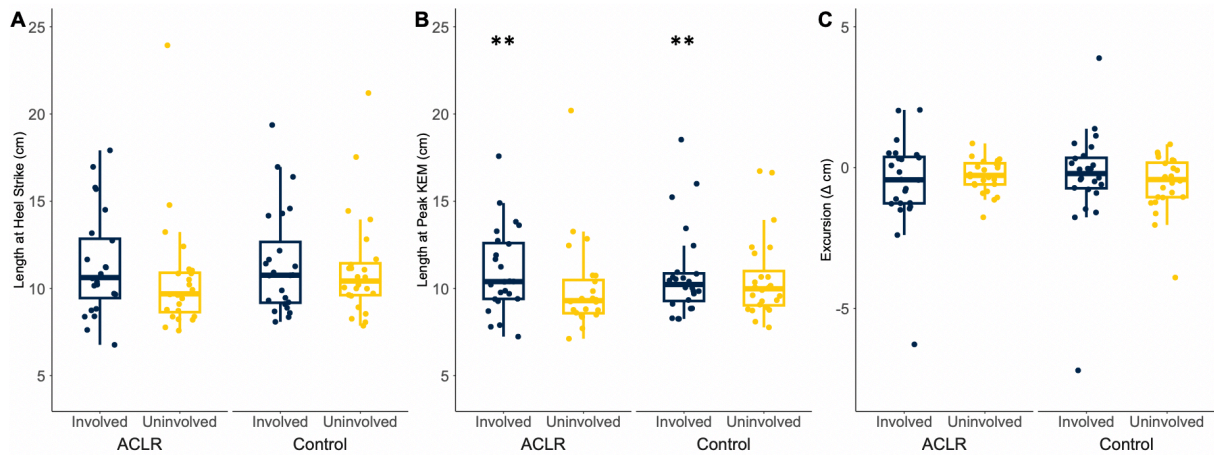
Figure 12. Fascicle Length Outcome by Group and Limb. No significant group by limb interactions were identified (p > 0.05) for fascicle length at heel strike (Panel A), fascicle length at peak KEM (Panel B) or fascicle length excursion (Panel C). However, there was a significant main effect for limb at peak KEM, where the involved limbs had longer fascicle lengths than the uninvolved limbs, independent of group (Panel B denoted by **). KEM indicates knee extension moment.
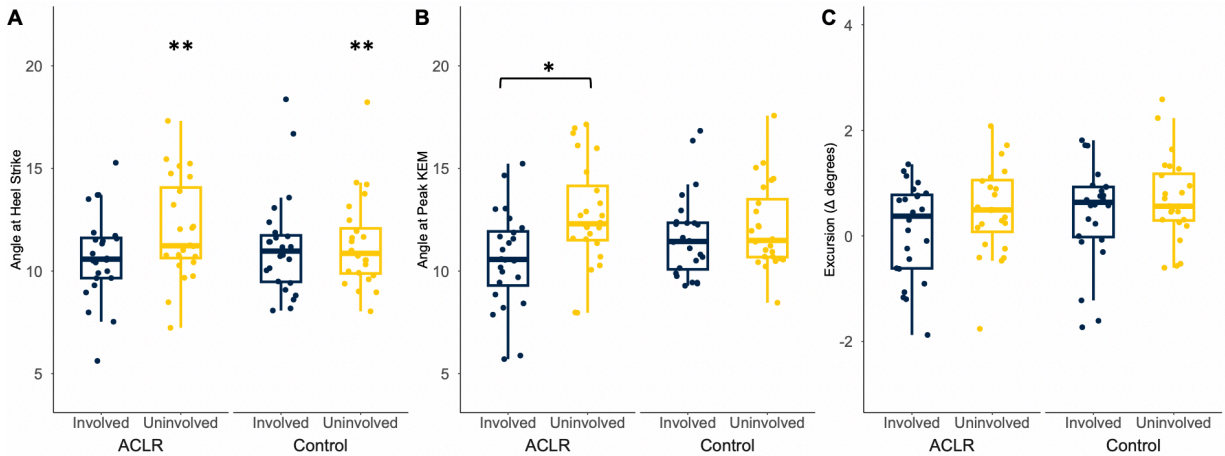
Figure 13. Fascicle Angle Outcome by Group and Limb. No significant group by limb interactions were identified between for fascicle angle excursion (Panel C). However, there was a significant main effect for limb at heel strike, where the involved limbs had smaller fascicle angles relative to the uninvolved limbs, independent of group (Panel A). In addition, there was a significant group by limb interaction at peak KEM, where the ACLR-involved limb exhibited 2.14 degrees less than the ACLR-uninvolved limb. (Panel B). KEM indicates knee extension moment; **, significant main effect of limb, independent of group p < 0.05; *, significant difference between limbs identified post hoc in the presence of a significant group by limb interaction, p < 0.05.

4.4.3 Fat-cleared Muscle Volume and Intramuscular Fat

VL volume and intramuscular fat outcomes are summarized in Table 7. There was a significant interaction between group and limb for fat-cleared muscle volume ($p < 0.001$). Post hoc analyses revealed that the ACLR-involved limb was on average 669 mm^3/kg or 7% smaller than the ACLR-uninvolved limb ($p < 0.001$), but no differences were noted between limbs for the Control group ($p = 0.502$). There were no significant interactions between group and limb, nor were there any main effects for intramuscular fat ($p > 0.05$).

	ACLR Involved	ACLR Uninvolved	Control Involved	Control Uninvolved	t	p value
Normalized Volume (mm^3/kg)	**8875 ± 1651**	**9576 ± 1530**	9428 ± 1888	9342 ± 2107	-3.93	<0.001*
Fat-cleared Volume (mm^3/kg)	**8729 ± 1615**	**9398 ± 1521**	9271 ± 1946	9180 ± 2092	-4.00	<0.001*
Intramuscular Fat (%)	1.64 ± 1.26	1.90 ± 1.12	1.85 ± 1.43	1.81 ± 1.25	-0.62	0.536

Table 7. Vastus Lateralis Muscle Characteristics. Data are reported as mean ± standard deviation. t statistics and p values reported are results of the limb by group interaction from the linear mixed effects model, with * denoting a statistically interaction at the level of p<0.05. Bold text indicates that post-hoc analyses revealed that the significant limb by group interaction stemmed from between-limb differences within the group ($p < 0.05$).

4.4.4 Knee Mechanics

Knee mechanics are summarized in Table 8 and Figure 14. There was a significant group by limb interaction for KFE. Post hoc analyses revealed that the ACLR-involved limb underwent 2.26 degrees or 15% less KFE than the ACLR-uninvolved limb ($p < 0.001$), but no differences were noted between limbs for the Control group ($p = 0.647$). There was not a significant group by

limb interaction for I) peak KEM II) peak power absorption or III) RMD. However, there were significant main effects of group that indicated that the ACLR group had on average 16% smaller peak KEM, 26% less peak power absorption, and 18% slower RMD compared to the Control group (p = 0.003-0.040; Figure 14).

	ACLR Involved	ACLR Uninvolved	Control Involved	Control Uninvolved	t	p value
Peak KEM$^{\gamma}$ (Nm/kg)	0.27 ± 0.16	0.32 ± 0.13	0.36 ± 0.12	0.34 ± 0.14	-1.44	0.156
RMD$^{\gamma}$ (Nm/ms*kg)	4.97 ± 1.18	5.46 ± 1.60	6.55 ± 2.08	6.19 ± 1.99	-1.48	0.146
KFE (°)	**12.81 ± 4.58**	**15.06 ± 3.08**	16.30 ± 2.78	16.01 ± 3.55	-2.87	0.006*
Peak Power Absorption$^{\gamma}$ (W/kg)	-0.50 ± 0.39	-0.65 ± 0.43	-0.79 ± 0.39	-0.76 ± 0.40	1.72	0.092

Table 8. Knee Mechanics. Data are reported as mean ± standard deviation. t statistics and p values reported are results of the limb by group interaction from the linear mixed effects model, with * denoting a statistically interaction at the level of p<0.05. Bold text indicates that post-hoc analyses revealed that the significant limb by group interaction stemmed from between-limb differences within the group (p < 0.05). $^{\gamma}$ denotes a significant main effect of group where the Control group had an increased peak KEM relative to the ACLR group. Abbreviations: KEM = Knee Extensor Moment; RMD = Instantaneous Rate of Moment Development; KFE = Knee Flexion Excursion.
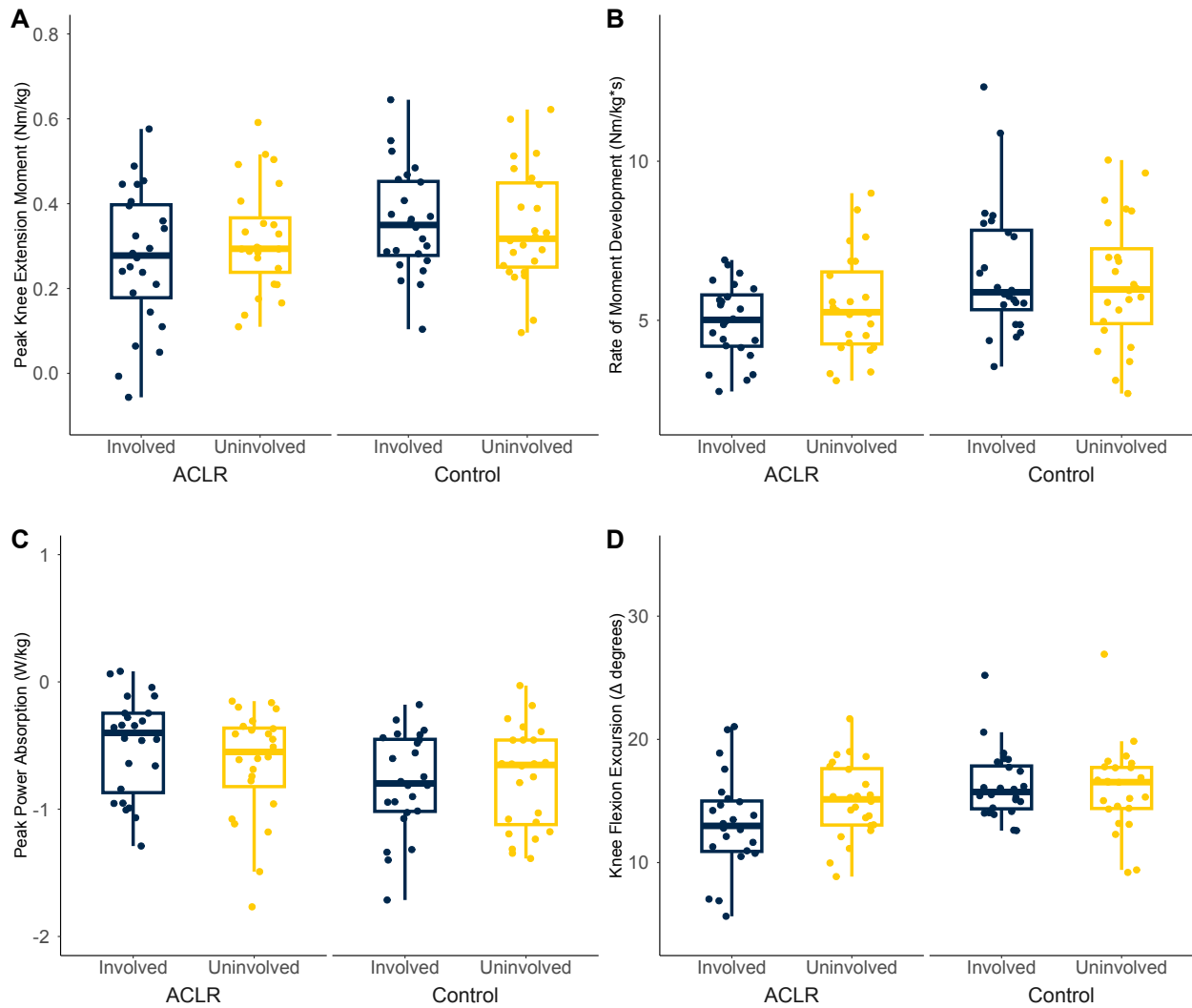
Figure 14. Knee Mechanics by Group and Limb. There was a significant group by limb interaction for KFE that revealed the ACLR-involved limb underwent 15% less KFE than the ACLR-uninvolved limb (Panel D). No significant group by limb interactions were identified for peak KEM, peak power absorption, or RMD (Panels A-C). However, there were significant main effects of group that indicated that the ACLR group had on average 16% smaller peak KEM, 26% less peak power absorption, and 18% slower RMD compared to the Control group (Panels A-C). KFE indicates knee flexion excursion; KEM, knee extension moment, RMD, instantaneous rate of moment development; **, significant main effect of group $p < 0.05$; *, significant difference between limbs identified post hoc in the presence of a significant group by limb interaction, $p < 0.05$

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4.4.5 Associations Between Fascicle Mechanics and Intramuscular Fat

No significant interactions were observed when examining the associations between intramuscular fat and fascicle mechanics (including fascicle length and angle excursion, fascicle length and angle at HS and at peak KEM; $p > 0.05$; Figure 15).
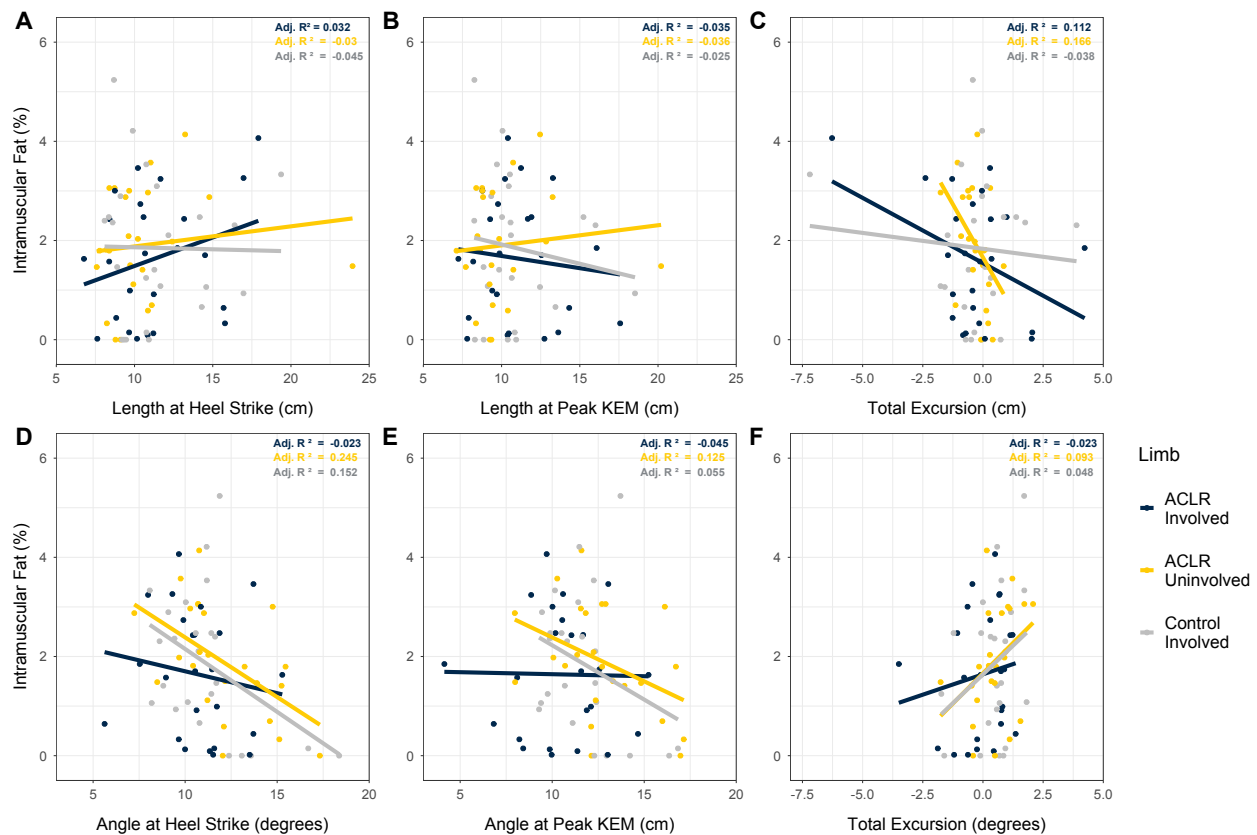
Figure 15. Associations Between of Intramuscular Fat and Fascicle Length (Panels A, B, C) and Fascicle Angle Mechanics (Panels D, E, F). Blue data points and regression lines indicate ACLR-involved limb data. Yellow data points and regression lines indicate ACLR-uninvolved limb data. Gray data points and regression lines indicate Control-involved limb data. No significant interactions ($p > 0.05$) were identified between limbs for the associations of intramuscular fat and fascicle length at heel strike (Panel A), fascicle length at peak KEM (Panel B), total fascicle length excursion (Panel C), fascicle angle at heel strike (Panel D), fascicle angle at peak KEM (Panel E), and total fascicle angle excursion (Panel F). KEM indicates knee extensor moment.

4.4.6 Associations Between Fascicle Angle at Peak KEM and Knee Mechanics

There was a significant interaction for the association of RMD and fascicle angle between the ACLR-involved and Control-involved limbs (ß = -0.92, t[56] = -2.23, p = 0.020; Figure 6B). Post hoc analyses revealed that a significant positive association between RMD and fascicle angle and in the ACLR-involved limb (ß = 0.91, p = 0.01) but not the Control-involved limb (ß = -0.01, p = 0.96). Separate regression analyses revealed that RMD accounted for 20% of the variance in fascicle angle (adjusted R-square = 0.16) in the ACLR-involved limb with overall model significance (F[1,22] = 5.45, p = 0.029).

No significant interactions were determined between fascicle angle and peak power absorption (p =0.168 – 0.727) or KFE (p =0.157 – 0.696).
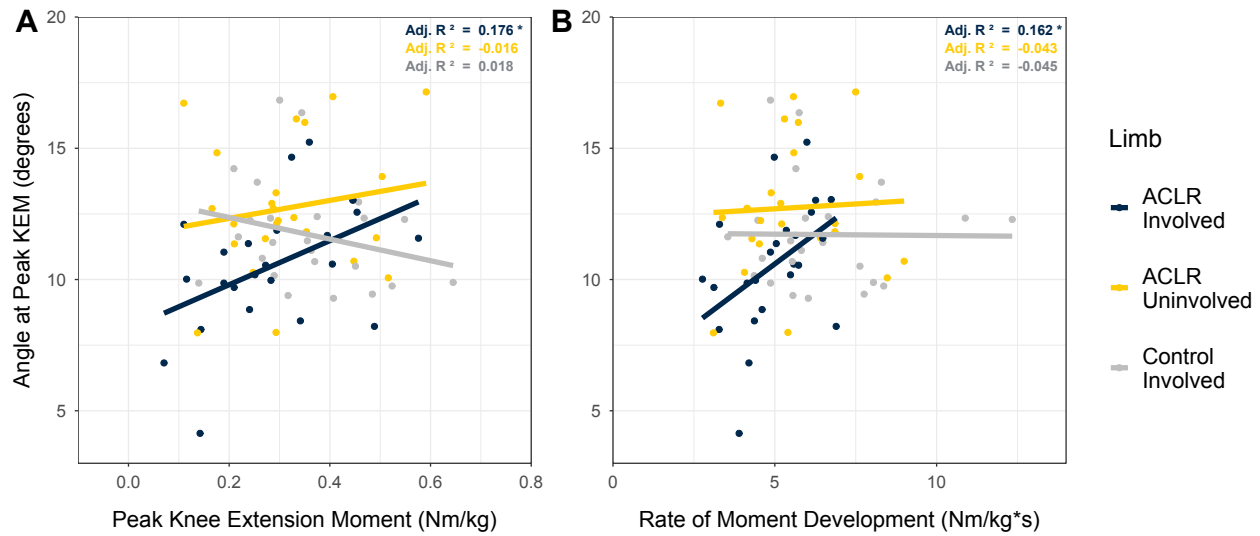
Figure 16. Associations Between Fascicle Angle at Peak KEM and Peak KEM (Panel A) and RMD (Panel B). Blue data points and regression lines indicate ACLR-involved limb data. Yellow data points and regression lines indicate ACLR-uninvolved limb data. Gray data points and regression lines indicate Control-involved limb data. There was a significant interaction between the ACLR-involved and Control-involved limb for the associations of fascicle angle at peak KEM and peak KEM (Panel A; p = 0.031). Post hoc analyses revealed the association was significant in the ACLR-involved limb (p = 0.03) but not the Control-involved limb (p = 0.29). Peak KEM accounted for 18% of variance of fascicle angle in the ACLR limb (Panel A). There was a significant interaction between the ACLR-involved and Control-involved limb for the associations of fascicle angle at peak KEM and RMD (Panel B; p = 0.020). Post hoc analyses revealed the association was significant in the ACLR-involved limb (p = 0.01) but not the Control-involved limb (p = 0.96). RMD accounted for 16% of variance in fascicle angle in the ACLR limb (Panel B). KEM indicates knee extensor moment; RMD; rate of moment development.

4.5   Discussion

The main objectives of our study were to I) comprehensively evaluate if fascicle mechanics are altered in those with a protracted history of ACLR, II) determine if fascicle mechanics could be explained by intramuscular fat, and III) explore the associations between fascicle and knee mechanics. We hypothesized that individuals with a chronic history of ACLR would exhibit less fascicle length and angle excursions compared to healthy controls. In contradiction to our hypotheses, we found a lack of significant differences in fascicle length and angle excursions between groups. However, we identified that the ACLR-involved limb demonstrated a smaller fascicle angle at the instant of peak KEM (Figure 13B). These data indicate that after ACLR, a

muscle's overall change in shape (fascicle length and angle measured as excursion) is similar to healthy controls, however, there are fascicle angle deficits that occur when greatest demand is placed on the quadriceps muscle (e.g., peak KEM). We also hypothesized that the changes observed in fascicle mechanics could be explained by increases in intramuscular fat in the ACLR group. Our hypothesis was not supported as differences in the quantity of intramuscular fat or associations between intramuscular fat and fascicle outcomes compared to the Control group were not identified. We also observed positive associations between fascicle angle at peak KEM and peak KEM and the RMD, helping to illuminate important factors in the complex inter-play between muscle and knee mechanics that may precipitate joint degeneration but warrant further investigation. This study is the first study to report an in vivo assessment of a comprehensive fascicle excursion outcome (e.g., including overall excursion measured at every frame) throughout weight acceptance at a chronic time point following ACLR. It is also the first to comprehensively assess intramuscular fat post-ACLR using MRI analyses.

Our findings contribute to the growing body of research on the complex nature of global muscle dysfunction after ACLR. They highlight that quadriceps weakness, neurological impairments, and whole muscle atrophy alone cannot fully explain the extent of quadriceps dysfunction following ACLR. Our study identifies intrinsic factors that contribute to muscle dysfunction, persisting into the chronic stages of recovery that includes a decrease in fascicle angle at peak KEM. Notably, these findings were observed in the absence of any strength deficits, providing further evidence to support strength recovery is just one aspect of rehabilitation and other components of muscle function should be considered. Our observation of a decrease in fascicle angle aligns with the findings of Munsch et al., who reported a reduction in fascicle angle between heel strike and peak KEM in patients within 12 months post-ACLR.[63] Collectively, our

study and those of Munsch et al. provide evidence of diminished fascicle angle at peak KEM during walking in both the acute (< 12 month) and chronic (>18 months) stages of ACLR recovery. It should be noted that we did observe a main effect for limb at HS, indicating that the uninvolved limbs had an increased fascicle angle, independent of group. Though significant, this difference was small (Table 6; Figure 13) and may have been influenced by our choice to randomly assign Control group right or left limbs to involved or uninvolved. Larger sample sizes and improved processing techniques may help uncover the origin of this finding. In contrast to Munsch et al., we revealed that the decrease in fascicle angle at peak KEM in the ACLR-involved limb coincided with preserved fascicle length at specific time points in stance (HS and peak KEM) and fascicle length excursion throughout weight acceptance (Table 6; Figure 12). This implies that the irregular fascicle lengths observed in the early stages following ACLR may gradually normalize over time and may not be the root cause of the observed changes in knee mechanics during walking in later stages of recovery. Due to the limited information available from only two cross-sectional studies evaluating fascicle length in vivo post-ACLR, the field would greatly benefit from incorporating longitudinal assessments of muscle mechanics throughout recovery following ACLR.

Fascicle angle plays a key role in the maximal force generating capacity of a muscle as larger muscles typically have greater fascicle angles and produce more force. However, this understanding applies to a muscle's architecture at rest and how much force can be produced during stationary movements and therefore, does not consider a muscle's size and composition, or its ability to change shape and produce force during movement. A loss in muscle size (i.e., atrophy), tends to result in smaller fascicle angles as the remaining fibers align in a more parallel arrangement. Despite this tendency, we found that muscle size in the ACLR limb was not related to the decrease in fascicle angle ($F[1,46] = 2.21$, $p = 0.144$). However, muscle size alone does not

capture the ratio of contractile and non-contractile elements, which may be reflected by changes in tissue composition and help to explain the decrease in fascicle angle. As we also found that intramuscular fat did not contribute to abnormalities in fascicle mechanics, we propose that other factors of muscle composition may be responsible for the observed changes in fascicle angle. Fibrotic tissue is a non-contractile element that has the potential to interfere with both fascicle angle and force transmission. While force transmission is often discussed in terms of longitudinal transfer from fascicles to tendons[70] lateral force transmission between myofibrils and ECM components[251] can account for more than 50% of force production.[252,253] The observed differences in fascicle angle and knee joint loading between the Control and ACLR groups may suggest the involvement of lateral force transmission mechanisms. Current two-dimensional in-vivo ultrasound primarily captures longitudinal force transmission, and our study focused on intramuscular fat, potentially missing compositional changes beyond fat such as fibrotic tissue development that has the potential to affect both longitudinal and lateral force transmission. Fibrotic tissue can be observed through expansions of the ECM (e.g., collagen) and can alter a muscle's passive stiffness through compromised gliding between layers of collagenous tissue in the ECM, in part, due to myofibrillar disorganization.[79] This not only reduces the relative percentage of contractile elements of the muscle, but can also induce deformation and restrict longitudinal and lateral force transmission.[81,82,251-253] While our study did not capture these compositional changes, previous research has been reported expansions of the ECM and increases in FAPs following ACL injury.[37,40] Therefore, further research exploring both muscle atrophy and composition (e.g., fibrotic tissue) following ACLR is necessary to understand its prevalence and functional impact, which may go undetected with simplified measurements of muscle size and function (e.g., strength).

The changes in fascicle angle observed in our study may also be a result of altered knee mechanics. Patients who underwent ACLR showed decreased peak KEM, slower instantaneous RMD, and reduced peak power absorption compared to control limbs. These findings align with previous reports of underloading in the injured limb following ACLR.[166,254-256] Abnormal loading has been associated with a host of degenerative joint changes including changes in the structure and composition of articular cartilage that precipitates the development of post-traumatic osteoarthritis.[254-257] Restoring normal walking patterns is often considered crucial to remediate degenerative joint changes and the high prognosis of post-traumatic osteoarthritis following ACLR. However, no other study has investigated the relationships between muscle architecture and loading mechanics (e.g., walking patterns) at chronic time points following ACLR to date. As such, the identified abnormality in fascicle angle that occurred specifically during the peak demand of quadriceps force and was associated with the magnitude (peak KEM) and rate of force produced (RMD) is an important finding as it implies that altered fascicle mechanics might be a contributing factor to the observed biomechanical irregularities during activities that require high levels of force and rapid force development. Understanding and addressing this relationship could help improve functional outcomes for individuals undergoing ACLR. The magnitude of force vs. rate of force development can have different clinical implications. It may be expected that a smaller fascicle angle permits more longitudinal force transmission and leads to a greater magnitude of force (i.e., increase load). However, this omits lateral force production which can contribute significantly to total muscle force production and may play a role in both the observed decrease in fascicle angle and peak KEM. Further research is needed to understand the role fascicle angle plays in both lateral and longitudinal force transmission and how it may impact joint loading. In addition, it would also be unsurprising that an atrophic muscle would produce less peak force. However, this only

occurred during walking through a surrogate measure of load (peak KEM) and was not captured during MVCs. In addition, this study only includes muscle size of the VL and not the other quadriceps muscles contributing to force output during MVC's or during walking. Regardless, the finding of decrease in fascicle angle suggests that there are functional impairments in how the VL contracts during activities of daily living, which may go undetected through MVC assessments. It is also important to acknowledge that our finding of a slower RMD in the ACLR suggests a deficit in how quickly force was produced. Notably, loading rates can also be influenced by the timing of muscle activation at the onset of contraction. Alterations in muscle activity such as early or delayed onset or smaller magnitudes have been previously demonstrated following ACLR.[258-260] There have also been a handful of reports on fascicle angle as a function of muscle activity that have shown a positive linear association between muscle activity and fascicle angle.[261,262] Based on this, it is reasonable to suggest that a smaller fascicle angle, peak KEM, and slower RMD may be a consequence of less muscle activity. While we didn't report electromyography data in this paper due to physiological and technological challenges in placing the probe and surface electromyography over the muscle belly, it is possible that these patients are unable to achieve adequate muscle activation which may impact peak KEM, RMD, and fascicle angle.

Given the deficits in fascicle angle at peak KEM and the associations between fascicle angle and knee joint loading in the ACLR-involved limb (Figure 16), our results suggest that interventional strategies aimed at restoring loading mechanics following ACLR may help restore proper fascicle mechanics.[234] Alternatively, it remains possible that abnormal fascicle mechanics drive alterations in walking mechanics. As muscles adapt to the level of load placed upon them, a reduction in joint loading may limit the functional demand on the muscle. While speculative, this repetitive reduction in load over time may facilitate changes at the muscle level (e.g., cellular, and

architecture), as seen in muscle disuse-atrophy.[263] Future longitudinal research examining these relationships is warranted to understand the relationship between fascicle mechanics and joint loading to identify muscular or biomechanical factors that can be targeted throughout rehab post-ACLR. Importantly, while the VL is the largest quadriceps contributor to sagittal plane loading, changes in the other quadriceps muscles including the vastus intermedius, vastus medialis, and rectus femoris have been reported and collectively, constitute global quadriceps dysfunction following ACLR.[99,264,265] Future work should consider the behavior of all the quadricep muscles as the VL may not reflect behavior of the other quadriceps muscles.[266,267]

It is important to recognize that fascicle mechanics influence force production and transmission to tendons during movement. Tendons are passive elements that store and release energy and have unique mechanical properties such as stiffness that can impact muscle-tendon unit function. Previous studies have shown in healthy limbs (controls or ACLR-uninvolved limbs) the predominant isometric or slightly concentric behavior of fascicles allows tendons to lengthen and therefore, contribute more to the overall length change of the muscle-tendon unit compared to fascicles themselves.[46,63] This can be thought of as a decoupling relationship between muscle fascicles and tendons, where the primary contributor to muscle-tendon unit length changes are tendons rather than muscle fascicles. While we did not identify any changes in fascicle length in the current study, it is plausible that disruptions in the decoupling behavior of fascicle-tendon dynamics may exist. It is also possible that following ACLR patients have a shift away from their optimal operating length on the force-length curve, which could interfere with fascicle-tendon dynamics. Notably, all ACLR participants in our study, as well as in the study by Munsch et al., received a bone-patellar-tendon-bone graft-type during ACLR surgery. Prior research has indicated that following a bone-patellar-tendon-bone, there are increases in cross-sectional area

and a 24% decrease in Young's modulus.[268] Therefore, alterations in the mechanical properties of the tendon as a response to graft harvest may interfere with fascicle-tendon dynamics. Further research is needed to investigate the long-term consequences of bone-patellar-tendon-bone grafts and how changes in a tendon's mechanical properties influence fascicle-tendon mechanics. Future research would also benefit from understanding fascicle-tendon mechanics following ACLR across different graft-types and establishment of patient-specific force-length curves to understand if there are shifts away from optimal lengths following ACLR that could be targeted in rehabilitation.

Several technological factors should be taken into consideration when interpreting the results of this study. Ultrasound data is known to be susceptible to movement artifacts which can induce variability in the data. To minimize inaccuracies caused by transducer movement, our group implemented a series of experimental enhancements at the data collection stage including I) probe-specific mounts to improve the contact between the ultrasound probe and the skin II) limiting our study population to individuals within a 28 kg/m^2 BMI range to mitigate the potential effects of subcutaneous fat and III) having a single lab personnel position the probe for all subjects. There are also many factors to consider during the data processing and analysis stage. Traditional approaches of measuring fascicle architecture and excursion are done at discrete points during stance (e.g., heel strike, peak KEM) or at rest, and often only include one representative fascicle. However, discrete moments in time give no indication into how the fascicle is behaving throughout movement and regional variations in fascicle architecture have been reported,[269,270] suggesting tracking multiple fascicles in a frame may improve the characterization of fascicle mechanics. Therefore, to improve data processing methods, we developed a rigorous processing pipeline utilizing open-sourced and custom software that tracks multiple fascicles and in every frame

throughout the duration of the movement. Despite these steps, certain outcomes (fascicle length at heel-strike and peak KEM) produced only moderate to good reliability scores. As such, we provide some caution in the interpretation of fascicle length at discrete points and suggest that the field would greatly benefit from standardizing data collections and processing methods to improve reliability and comparison across research sites.

In summary, we demonstrate that patients who underwent ACLR exhibit overall fascicle excursions (length and angle) and quantities of intramuscular fat that resemble those of healthy controls. However, we observed a smaller fascicle angle at peak KEM in the ACLR-involved limb, which coincided with a decrease in joint load measured by peak KEM and RMD. This indicates an abnormality in fascicle architecture specifically during the peak demand of quadriceps force and is associated with the magnitude and timing of force produced. Furthermore, this dysfunction existed despite full strength recovery. These findings add to the growing body of research that highlights the importance of considering intrinsic factors of muscle such as composition and mechanical behavior in evaluating muscle function.

4.6 Supplementary Material

		Cronbachs alpha
Fascicle Angle (degrees)	Heel Strike	0.832
	Peak KEM	0.848
	Excursion	0.969
Fascicle Length (cm)	Heel Strike	0.510
	Peak KEM	0.662
	Excursion	0.944

Table 9. Reliability Intraclass Correlations. A test-retest analysis of our custom-built application (MATLAB [version 2021a, The MathWorks, Natick, USA]) was performed by 1 rater on n = 4 limbs to assess the reliability of all fascicle outcomes including fascicle length and angle at heel strike and peak KEM, and fascicle length and angle excursions. Abbreviations: KEM = Knee Extensor Moment.

5 The Relationship Between Vasti Muscle Strength, Volume, and Intramuscular Fat with Quantitative MRI Metrics of Cartilage Health following ACL Reconstruction

5.1 Abstract

Persistent vasti muscle weakness following anterior cruciate ligament (ACL) injury is often at blame for the increased prevalence of post-traumatic osteoarthritis (PTOA) following ACL reconstruction (ACLR). However, muscle weakness constitutes merely a single facet of muscle function, overlooking the intrinsic properties of muscle, such as its composition, which could potentially impede strength recovery post-ACLR. For instance, intramuscular fat represents a non-contractile element that has been shown to be elevated in idiopathic osteoarthritis and may be interfering with strength recovery following ACLR and precipitating early joint degeneration. Understanding these multifaceted aspects of muscle is crucial for comprehensively addressing the recovery challenges after ACLR and for devising effective strategies to mitigate potential impediments to joint health. As such, our main objectives were to I) characterize vasti intramuscular fat and cartilage composition utilizing $T_{1\rho}$ and T_2 in whole and sub compartments with those with a history of ACLR II) determine the associations between intramuscular fat, muscle volume, and isometric strength and III) determine the association between muscle strength, size, and composition with $T_{1\rho}$ and T_2 imaging of articular cartilage as early indicators of cartilage composition following ACLR. While controlling for sex and BMI, linear mixed effects models were used to assess (I) quadriceps strength, (II) intramuscular fat and fat-cleared muscle volume and (III) $T_{1\rho}$ and T_2. Stepwise regressions were run to determine if I)

total vasti intramuscular fat or muscle volume was associated with strength or II) $T_{1\rho}$ and T_2. We demonstrate that patients with history of ACLR exhibit muscle atrophy of all vasti muscles, where the involved limb was 7-9% smaller than the uninvolved limb ($p < 0.05$). No significant group or limb differences were identified for intramuscular fat ($p = .420$-.999). Despite a significant group by limb interaction for quadriceps strength ($p = 0.049$), post-hoc analyses revealed no between limb differences ($p > 0.05$). Those with a history of ACLR also revealed changes in cartilage composition that primarily resided in the medial patella ($p = 0.047$), and anterior ($p = 0.037$) and posterior femur ($p = 0.021$). In ACLR- and Control-involved limbs, intramuscular fat nor quadriceps volume were associated with quadriceps strength ($p = 0.092 - 0.219$). In the ACLR-involved limb, vasti intramuscular fat explained 25-26% of variance in $T_{1\rho}$ in the in medial and lateral patella ($p = 0.013$-0.016). This was consistent in the Control-involved limb, where vasti intramuscular fat explained 26% of variance in $T_{1\rho}$ in the medial patella ($p = 0.013$) but not lateral patella ($p > 0.05$). No other associations were identified between intramuscular fat, quadriceps strength, or muscle volume in any other $T_{1\rho}$ and T_2 region of interest ($p > 0.05$) We demonstrate that patients with history of ACLR exhibit muscle atrophy and early disease-related changes in cartilage composition that cannot be explained by muscle strength, size, or intramuscular fat.

5.2   Introduction

Following anterior cruciate ligament (ACL) injury, patients generally opt to undergo ACL reconstruction (ACLR) to restore the mechanical stability of the joint. Surgical reconstruction is then followed by rigorous and prolonged rehabilitation ($\geq$6 months) in order to return patients to physical activity. Despite this standard of care, patients often encounter impairments such as persistent quadriceps weakness and an elevated risk of developing early onset post-traumatic

osteoarthritis (PTOA),[3] which has been reported on average in 50% of patients within 10-15 years following ACLR.[3] One of the predominant theories of PTOA development following ACL injury is that quadriceps weakness diminishes the quadriceps' capacity to absorb shock, altering joint biomechanics and the distribution of load across articular cartilage.[170] The change in load alters the balance of turnover of the extracellular components that comprise articular cartilage and consequently, may represent one factor initiating PTOA.[170] As such, research aimed at understanding the sources underlying quadriceps muscle weakness is critical to identify therapeutic targets for intervention to mitigate or prevent PTOA development.

In those with idiopathic osteoarthritis (i.e., osteoarthritis developing in knees without joint injury), it has been well documented that quadriceps weakness is a risk factor for the development and progression of the disease.[176-180,182,271] In contrast to this, data directly linking quadriceps weakness and PTOA development after ACL injury is sparse.[24,181] Keays et al[181] showed quadriceps strength outcomes (e.g., knee extensor torque, quadricep:hamstring ratio) were able to distinguish patients with tibiofemoral PTOA (measured by the Kellgren-Lawrence grading system) from those without following ACLR.[151] Further, Tourville et al[24] showed that lesser quadriceps strength prior to ACLR was associated with greater joint space width narrowing at 4 years post ACLR. Arhos et al[272] showed that greater limb symmetry in quadriceps strength was associated with lower odds of clinical PTOA as measured by self-reported function at 5 years post-ACLR. While these data provide support that muscle weakness is linked with indices of PTOA development after ACLR, the use of radiographs and assessments of joint space width narrowing are only reflective of changes in late-stage disease, wherein significant structural deterioration has already occurred and is largely considered irreversible. Alternatively, more advanced MRI techniques like $T_{1\rho}$ and T_2 can provide a more direct assessment of collagen orientation, and

proteoglycan and water concentration that are indicative of some of the earliest PTOA-related alterations in articular cartilage that precede structural change. For instance, recent work by Pietrosimone et al[273] has connected early quadriceps weakness after ACLR with longer $T_{1\rho}$ relaxations times (i.e., lesser proteoglycan density) in the surgical knee 6-months post-operatively. Wellsandt et al has also shown knee joint underloading was associated with longer T_2 relaxation times (i.e., greater collagen disorganization) in the surgical knee 1-month after ACL injury. As such, the use of sensitive MRI metrics such as $T_{1\rho}/T_2$ may aid in improving our understanding of the link between quadriceps function and PTOA after ACLR by elucidating early changes in articular cartilage composition that may be amenable to interventions.

In general, most studies investigating quadriceps function and osteoarthritis outcomes often focus on whole muscle metrics (e.g., isometric, and isokinetic strength,[24] volume,[274] cross-sectional area[275]) and indirect assessments of cartilage health (e.g., radiography[24] and clinical grading systems[181]). Importantly, other factors known to compromise quadriceps muscle tissue in idiopathic osteoarthritis such as non-contractile tissue (e.g., fibrosis[39,45] and intramuscular fat[98]), have largely been overlooked following ACLR, but may help to explain the relationship more fully between quadriceps dysfunction and PTOA. We are particularly motivated to investigate the influence of intramuscular fat as it resides in the extracellular matrix (ECM), which provides structural support for many cellular processes and is essential for adequate force transmission.[225] When there is an accumulation of intramuscular fat, it can restrict force transmission between myofibrils, aponeuroses, and the tendon.[80-82,225] Following ACL injury, it has been shown that the ECM expands due to collagen accumulation which occurs alongside elevated quantities of fibro-adipogenic progenitors (FAPs). This is important as FAP's can disrupt ECM remodeling and promote differentiation into fat and fibrotic tissue,[37,38,40] which has been associated with

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quadriceps weakness in other populations[276-278] and found to be a better predictor of knee osteoarthritis than muscle size alone.[98] Following ACLR, others[99] have shown increases in quadriceps intramuscular fat using B-mode ultrasound imaging. While important, B-mode ultrasound assesses intramuscular fat via echo intensity, which can be confounded by factors such as subcutaneous fat overlying muscle. Alternatively, mDixon magnetic resonance imaging (MRI) is a reliable imaging technique that enables the separation of fat and water through their unique chemical-shift properties, offering a non-invasive approach to assess fat accumulation more comprehensively in three-dimensions.[279,280] While this imaging modality has been utilized in other populations[281,282], it has not yet been performed following ACLR. Given the associations between intramuscular fat, quadriceps weakness, and osteoarthritis,[37,40,98,273] it remains plausible that intramuscular fat may interfere with quadriceps strength recovery and precipitate early joint degeneration following ACLR.

In order to determine the extent of intramuscular fat infiltration and disease-related changes in cartilage composition, we first characterized intramuscular fat in the vasti muscles both globally and regionally, and of $T_{1\rho}$ and T_2 in whole and sub compartments of those with a history of ACLR and healthy controls. We also explored if intramuscular fat and fat-cleared muscle volume were related to isometric strength in the ACLR and Control limbs. After characterizing independent factors of muscle and cartilage, we sought to determine the relationship between muscle strength, size, and composition with $T_{1\rho}/T_2$ imaging of articular cartilage as early indicators of cartilage composition following ACLR. We hypothesized that an increase in intramuscular fat (e.g., fat fraction), weaker isometric strength, and smaller fat-cleared muscle volume would correlate with more severe degenerative cartilage outcomes.

5.3 Methods

5.3.1 Experimental Design and Participants

Twenty-four individuals who had undergone primary unilateral ACLR were recruited from the Department of Orthopaedic Surgery and the general student population at the University of Michigan (Age: 22.8 ± 3.6 years, Time since surgery: 3.3 ± 0.09 years). ACLR individuals were eligible for this study if they: 1) were between 14-45 years of age, 2) had no prior knee injury or surgery other than current ACL 3) were between 1.5-5 years post-ACLR 4) had received a bone patellar tendon bone autograft and 5) had a body mass index (BMI) under 28 kg/m^2. ACLR participants were excluded if they had multiple ACLR's unilaterally or bilaterally. To characterize healthy profiles, a convenience cohort of twenty-four healthy individuals were also recruited (Age: 22.0 ± 3.1 years) that had no history of lower extremity injury or surgery and had a BMI under 28 kg/m^2. Demographic information for included participants can be found in Table 10.

All participants meeting these criteria underwent two experimental sessions over two days. The first experimental session consisted of patient reported outcomes and strength testing, while the second experimental session consisted of magnetic resonance imaging (MRI) to measure quadriceps fat infiltration via mDixon MRI sequence and cartilage proteoglycan, water, and collagen content via $T_{1\rho}/T_2$. mDixon scans were always collected first to allow a 30-minute unloading period for all cartilage imaging. All participants provided informed consent, and all protocols were approved by the University of Michigan Medical School Institutional Review Board (IRBMED: HUM00169174).

5.3.2 Patient Reported Outcomes

Participants completed a series of questionnaires to evaluate self-reported function including the Tegner Physical Activity,[229] International Knee Documentation Committee (IKDC)[230] and Knee Injury and Osteoarthritis Outcome Score (KOOS).[231]

5.3.3 Isometric Strength

Isometric knee extensor strength was assessed as previously described using an isokinetic dynamometer (CSMi Humac Norms Stoughton, MA).[232] Participants were provided strong verbal encouragement and real-time visual feedback to encourage maximal effort during all testing contractions.

The knee joint was positioned at 60° of flexion.[233] Participants then completed a standardized warm-up protocol consisting of two practice repetitions at each of the following intensities: 25%, 50%, and 75% of perceived maximal effort. Following these warm-up contractions, participants completed one practice trial at 100% of their perceived maximal effort. The maximal voluntary knee extensor torque during this final practice trial was then used to set a visual torque target for the subsequent MVC assessments. For test trials, all participants performed three MVC's in which they were instructed to kick out as hard as they could. Participants were given two minutes of rest between each trial. Peak knee extension (KE) torque (N·m) was extracted from each MVC trial. Peak torque was normalized to body mass (N·m/kg) and the peak of peaks was used in the subsequent analyses.

5.3.4 Intramuscular Fat and Muscle Volume

MRI data of the bilateral upper thighs were acquired with a 3-Tesla MRI Philips Ingenia scanner using a 16-element anterior torso coil and 12-element receive coil located within the table

coil. Three stacks were acquired with a 30-mm overlap covering a field of view (FOV) of 420 x 284 x 140 mm^3. Stack-specific shimming was applied independently to each stack to minimize B0 field inhomogeneity. An axial mDixon Quant sequence was performed with the following key acquisition parameters: 3D gradient echo; number of echoes = 6 (TE1 = 1.2 ms, delta TE = 0.9 ms); TR = 7.283 ms; flip angle = 3°; acquired matrix size = 264 x 178 mm; reconstructed matrix size = 288 x 288 mm; acquired voxel size = 1.6 x 1.6 x 2.2 mm^3; reconstructed voxel size = 1.3 x 1.3 x 1.1 mm^3; parallel imaging factor SENSE = 2; number of slices/stack = 740.

Post-processing was conducted via custom written MATLAB code. Five slices on the proximal and distal ends of each stack were removed to improve signal inhomogeneity that occurs at the ends of each stack. In the regions of overlap, slices closest to the center of the stack were retained. Stacks were then stitched together to generate fat only, water only, in phase, out of phase, and fat fraction images. To reduce the number of slices and maintain the signal-to-noise ratio from the acquisition, signals were averaged over every four slices. The whole vastus lateralis (VL), vastus intermedius (VI), vastus medialis (VM), and the femur were segmented on a limited number of slices on the water-only image and a semi-automatic method using a combination of diffeomorphic registrations was used to propagate these segmentations to the remaining slices (Figures 17A, 17B).[242,243] Propagations were refined in ITK snap.[244] Whole muscle volumes were extracted from the full region of interest. As subject mass and height were correlated with each other (r < 0.799), and muscle volumes were normalized to the subject mass. Custom-written software was then used to erode a single voxel border from all whole muscle segmentations and to split the whole muscles into sub-regions of interests (ROIs). Proximal, central, and distal sub-ROIs were created by splitting each muscle into 3 equal lengths in the proximo-distal direction (Figure 17C). To further quantify the extent of local damage, segmentations of each muscle were

partitioned into two depth levels. Deep and superficial sub-ROIs were discriminated using the distance from the femur gravity center as previously described (Figure 17D).[283] Fat-cleared muscle volume was computed by subtracting the median fat fraction from the normalized muscle volume.[245] Muscle volume was reported descriptively, while fat-cleared muscle volume was used in subsequent statistical analyses. The distribution of intramuscular fat was also evaluated descriptively as a function of muscle length from the distal to proximal axis as previously described.[284] Briefly, slice numbers were expressed as a percentage of muscle length where the most distal slice was set to 0% and the most proximal slice was set to 100%, and values were log transformed to visualize between-limb variances (Figure 19).
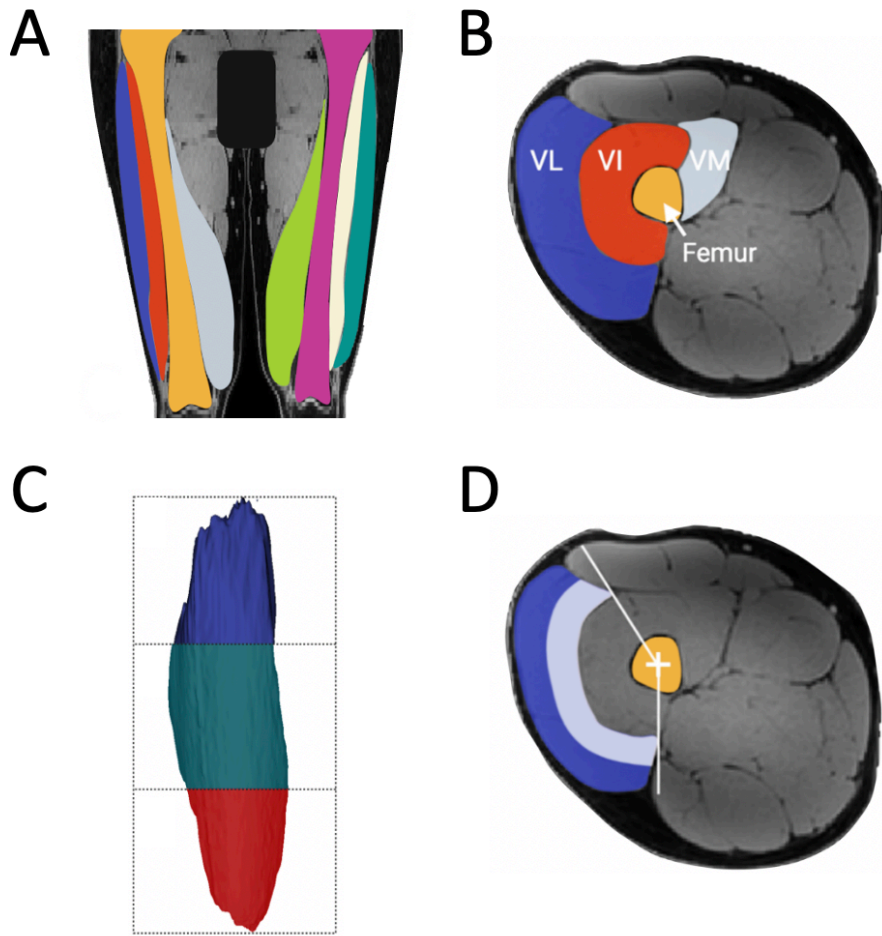
Figure 17. Representative Muscle Segmentations Panels A and B represent whole muscle regions of interest (ROI) in the frontal (A) and axial (B) planes. VL indicates Vastus Lateralis; VI, Vastus Intermedius; VM, Vastus Medialis. Panel C represents an example of the proximal (top), central (middle), and distal (bottom) sub-ROI's for the VL where the muscle was split into 3 equal lengths in proximo-distal direction. Panel D represents an example of the superficial and deep muscle sub-ROIs for the VL, where the muscle was partitioned to two different depths using the distance from the femur gravity center.

5.3.5 Cartilage Imaging Protocol

$T_{1\rho}$ and T_2 mapping of both knees was acquired with a 3-Tesla MRI Philips Ingenia scanner using a 16-element T/R knee coil. Sagittal plane 3D images (field of view = 128 x 128 x 128 mm^3) with different spin-echo times (TEs) or spin-lock durations (SLs) were acquired as previously described.[285] In brief, $T_{1\rho}$ weighted images were acquired in elliptic-centric k-space with the following key acquisition parameters: 3D turbo gradient echo; TE = 6.1 ms; TR = 7.283 ms; flip angle = 10°; acquired voxel size = 0.40 x 0.40 x 3.00 mm^3; reconstructed voxel size = 0.24 x 0.24 x 3.00 mm^3; parallel imaging factor SENSE = 2. The spin-lock RF field strength was 500 Hz and the SL durations were 0, 10, 20, 30, and 40 ms. T_2 images were acquired in an interleaved multislice multiecho sequence with the following key acquisition parameters: 3D turbo spin-echo; TR = 2500 ms; effective TEs = n* 6.1 with n = 1, 2, 3, 4, 5, 6, 7, 8; acquired voxel size = 0.60 x 0.60 x 3.00 mm^3; reconstructed voxel size = 0.24 x 0.24 x 3.00 mm; parallel imaging factor SENSE = 2.

The five $T_{1\rho}$ images were registered to one another using intra-registration and then co-registered to the T_2 weighted images using Elastix (http://elastix.bigr.nl/wiki)[286] based on previously described parameter settings for rigid transformation model.[158] $T_{1\rho}$ and T_2 parametric maps were generated by pixel-wise, nonlinear least squares based on an exponential relaxation decay model, $S(t_i) = S_0 \exp(-t_i * P)$, where $P = 1/T_2$ or $1/T_{1\rho}$, and $t_{i=}$ effective TEs or SLs. Fit quality was measured by the goodness of fit, coefficient of determination (adjusted R^2 values) for each pixel. Pixels with R^2 values < .8 were excluded from the analysis.

5.3.6 Whole and Sub Compartment Cartilage Analysis

The first 3D volume of the co-registered T_2 (TE = 6.1) was segmented using Dragonfly Image Analysis Software (version 4.1; Object Research Systems). Anterior and posterior menisci, trochlea, patella, and tibial, and femoral cartilage were manually segmented. The patella cartilage was separated into the whole medial and lateral ROI's based on the apex of the patella. The tibial and femoral cartilage was divided into whole medial and lateral ROI's based on the location of the intercondylar notch (Figure 18A and 18B).

All whole ROIs (medial and lateral patella, femur, and tibia) and the anterior and posterior menisci were then exported into custom software written in MATLAB (version 2021a, The MathWorks, Natick, USA) to further partition the ROI's. (Figure 18C and 18D) The whole tibial and femoral ROIs were partitioned into regions based on the location of the menisci. The combined weight bearing ROI's we defined as the entire region between the anterior and posterior menisci horns, and could be considered the combined weight-bearing medial and lateral femur (cMF, cLF) and tibia (cMT, cLT).[287,288] The combined weight bearing ROIs was then separated into the medial and lateral anterior, central, and posterior femur (cMF-a, cMF-c, cMF-p, cLF-a, cLF-c, cLF-p) and tibia (cMT-a, cMT-c, cMT-p, cLT-a, cLT-c, cLT-p).[287,288] The non-weight bearing ROI's on the femur located outside of the anterior and posterior horns of the meniscus included the anterior and posterior femoral ROIs (aMF, aLF, pMF, pLF). Other ROI's included the medial and lateral patella, and trochlea. For each whole- and sub-ROI, $T_{1\rho}$ and T_2 relaxation times were computed bilaterally.
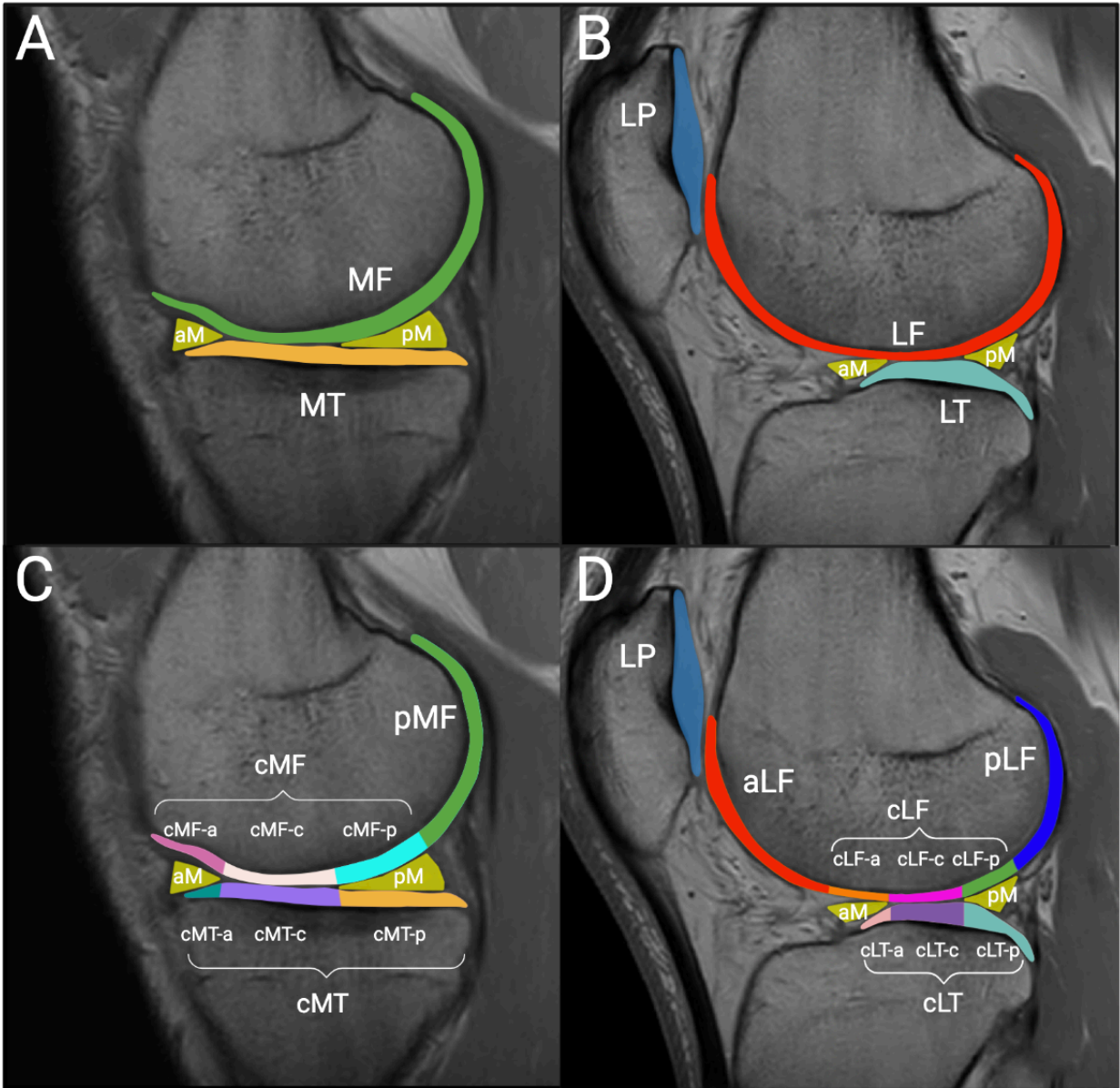
Figure 18. Examples of Whole and Sub Cartilage Regions of Interests (ROIs). Panels A is a representative medial image demonstrating the whole medial femur (MF) and tibia (MT). Panel B is a representative lateral image demonstrating the whole lateral femur (LF), tibia (LT), and patella (LP). Panel C is a representative image demonstrating medial sub-ROIs including the combined weight bearing sub-ROIs (cMF, cMT) and separated weight bearing sub-ROIs including the anterior, central, and posterior sub-ROI's of the femur (cMF-a, cMF-c, cMF-p) and tibia (cMT-a, cMT-c, cMT-p). Panel D is a representative image demonstrating lateral sub-ROIs including the combined weight bearing sub-ROIs (cLF, cLT) and separated weight bearing sub-ROIs including the anterior, central, and posterior sub-ROI's of the femur (cLF-a, cLF-c, cLF-p) and tibia (cLT-a, cLT-c, cLT-p). aM and pM in all panels A-D indicate anterior and posterior meniscus. Anterior and posterior medial (aMF, not pictured; pMF) and lateral femur (aLF; pLF) are also shown in panels C and D.

5.3.7 Statistical Analysis

At the time of this experimental design, pre-existing data on the associations between intramuscular muscle fat infiltration and proteoglycan density measured by $T_{1\rho}$ and collagen/water content measured by T_2 was not available. Therefore, sample size for the current study was determined using previously published work from Jungmann et al[289] that measured total extramuscular fat and osteoarthritis as quantified by the Kellgren and Lawrence grading system, the gold standard scoring system for diagnosing radiographic knee OA. After removing two outliers, there was an $R^2 = .23$ between extramuscular fat and Kellgren and Lawrence scores. Assuming a similar effect of muscle fat infiltration on cartilage composition as reported in the aforementioned study (effect size $|\rho| = 0.48$) and an alpha level and power set at 0.05 and 80% (1-beta), we estimated that a total of 23 participants with a history of ACLR were required to detect a significant association (G*Power v3.1.9.6).[246]

Statistical analyses were conducted using R Statistical Software (4.2.2, Vienna, Austria) with a significance level set at $p \leq 0.05$. Participant demographics and patient reported outcomes between the ACLR and Control groups were compared using independent t-tests (Table 10). To account for between-limb variability in the Control subjects, both Control limbs were included in subsequent linear mixed effect model analyses. Given that almost all of our subjects considered their right-leg as dominant (n=47 of 48) and 9 of 24 ACLR subjects received ACLR on their left limb, we randomly chose 9 Control subjects and assigned their left limb as involved, while the other Control subjects had their right limb assigned to involved. The limb that received surgery in the ACLR group was assigned to the ACLR-involved limb, while the contralateral limb was assigned to ACLR-uninvolved.

To characterize and compare intramuscular fat and $T_{1\rho}/T_2$ between ACLR and Controls, the following variables were analyzed using linear mixed effects models (lmer function from the lmerTest and lme4 packages in R[247,248]): (I) Strength, (II) total vasti intramuscular fat and fat-cleared muscle volume (III) global intramuscular fat and fat-cleared muscle volume of the VI, VL, and VM, (IV) intramuscular fat by region (proximal, central, distal, superficial, and deep) of the VI, VL, and VM, (V) and $T_{1\rho}/T_2$ of weight bearing and non-weight bearing ROI's. The fixed factors in the models included limb (involved vs. uninvolved), group (ACLR vs. Control), and their interaction. The interaction term compared the differences between limbs (injured-uninjured) for each group. Notably, there were cases where limbs did not have cartilage able to be visualized in the aMF. As such, the linear mixed effects model for this ROI only included 20 ACLR-involved, 21 ACLR-uninvolved, 23 Control-involved, and 16 Control-uninvolved limbs. To understand if intramuscular fat was distributed differently within limbs, we also ran linear mixed effects models for individual vasti muscles and compared the interaction of region by group by limb. Subject was treated as a random factor and sex and BMI were included as control variables in all linear mixed effects models. In the case of a significant group by limb or region by group by limb interactions, post hoc analyses were conducted using the emmeans package[249] to calculate estimated marginal means and perform pairwise comparisons between groups with Tukey adjustment for multiple comparisons. Separate regression analyses were also run to determine if time since surgery was associated with I) intramuscular fat, II) volume and III) strength, or IV) $T_{1\rho}/T_2$ in the ACLR-involved limb.

Separate stepwise regressions were run in the ACLR-involved (primary aim) and Control-involved limb (provided as supplementary material) to determine if total vasti intramuscular fat, fat-cleared muscle volume, and/or strength were associated with $T_{1\rho}/T_2$ relaxation times in the

whole ROIs (femur, patella) and combined weight-bearing ROIs (femur, tibia), while controlling for sex and BMI. The whole tibia ROI was treated as the combined weight-bearing ROI. In the cases where a significant association was determined between total vasti intramuscular fat and volume with $T_{1\rho}/T_2$ ROIs, we further explored if intramuscular fat and volume of individual vasti muscles (VL, VI, VM) were associated with $T_{1\rho}/T_2$ relaxation times. Separate stepwise regression analyses were also run in the ACLR-involved and Control-involved limb to determine if total vasti intramuscular fat or fat-cleared muscle volume was associated with strength, while controlling for sex and BMI.

5.4 Results

5.4.1 Patient Reported Outcomes

KOOS subscale and IKDC results are summarized in Table 10. The ACLR cohort had significantly worse outcomes on all KOOS subscales and IKDC knee-specific outcomes than the control cohort (p = <0.001 – 0.039).

	ACLR (n = 24)	Control (n = 24)	p value
Females/Males	15/9	14/10	-
Age (yrs)	22.8 ± 3.6	22.0 ± 3.1	0.406
Height (m)	1.70 ± 0.1	1.70 ± 0.1	0.856
Weight (kg)	68.7 ± 8.9	69.9 ± 13.6	0.732
Body Mass Index	23.2 ± 1.9	23.3 ± 2.6	0.801
Time after surgery (yr)	3.3 ± 0.9	N/A	-
Tegner Activity Scale	Pre: 8.4 ± 1.1 Post: 6.8 ± 1.6	Pre: 6.8 ± 1.3 Post: 6.7 ± 1.2	Pre: <0.001* Post: 0.815
KOOS Symptoms	84.7 ± 10.6	95.7 ± 5.4	<0.001*
KOOS Pain	95.4 ± 5.3	98.7 ± 2.3	0.007
KOOS Activities of Daily Living	99.0 ± 1.4	99.8 ± 0.9	0.039
KOOS Sports and Recreation	89.0 ± 8.5	98.3 ± 4.3	<0.001*
KOOS Quality of Life	83.1 ± 14.6	99.2 ± 2.1	<0.001*
IKDC	88.9 ± 8.8	98.7 ± 3.0	<0.001*

Table 10. Data are reported as mean ± standard deviation.*Denotes statistical significance at the level of $p<0.05$. Abbreviations: KOOS = Knee Injury and Osteoarthritis Outcome Score; IKDC = International Knee Documentation Committee

5.4.2 Muscle Strength, Volumes, and Intramuscular Fat

There was a significant group by limb interaction for isometric quadriceps strength. However, post-hoc analyses indicated the differences were small and non-significant ($p = 0.098$-0.249; Table 11). Total vasti and individual muscle volumes and intramuscular fat are summarized in Tables 12 and 13 respectively. There was a significant interaction between group and limb for total vasti fat-cleared volume ($p < 0.001$). Post hoc analyses revealed the vasti muscles in the ACLR-involved limb were on average 2007 mm^3/kg or 8% smaller than the vasti muscles in the ACLR-uninvolved limb. There were no significant interactions between group and limb, nor were there any main effects for vasti intramuscular fat ($p > 0.05$). These findings were consistent for what was found for individual vasti muscles (Table 4). There was a significant interaction between

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group and limb for the VI, VL, and VM fat-cleared volume ($p < 0.001$). Post hoc analysis revealed that the VI in the ACLR-involved limb was on 9% smaller (701 mm^3/kg) than the VI in the ACLR-uninvolved limb. Similarly, the VL in the ACLR-involved limb was 7% smaller (669 mm^3/kg) and the VM was 9% smaller (636 mm^3/kg) compared to the ACLR-uninvolved limb.

	ACLR Involved	ACLR Uninvolved	Control Involved	Control Uninvolved	t	p value
Peak Torque (N·m/kg)	2.96 ± 0.51	3.12 ± 0.45	3.13 ± 0.77	3.01 ± 0.68	-2.02	0.049*

Table 11. Isometric Quadriceps Strength by Limb. Data are reported as mean ± standard deviation. t statistic and p value reported is result of the limb by group interaction from the linear mixed effects model, with * denoting a statistical interaction at the level of $p<0.05$. Though the ACLR limb has a smaller mean, post-hoc analyses revealed a non-significant between-limb difference in both groups ($p < 0.05$).

	Involved ACLR	Uninvolved ACLR	Involved Control	Uninvolved Control	t	p value
Normalized Volume (mm^3/kg)	**22579 ± 3856**	**24636 ± 3617**	23189 ± 4906	23034 ± 5255	-5.56	<0.001*
Fat-cleared Volume (mm^3/kg)	**22212 ± 3796**	**24218 ± 3600**	22836 ± 5000	22669 ± 5255	-5.50	<0.001*
Intramuscular Fat (%)	1.65 ± 0.98	1.70 ± 1.00	1.65 ± 1.11	1.67 ± 1.00	-0.11	0.916

Table 12. Total Vasti Muscle Volume and Intramuscular Fat. Data are reported as mean ± standard deviation. t statistics and p values reported are results of the limb by group interaction from the linear mixed effects models, with * denoting a statistically interaction at the level of $p<0.05$. Bold text indicates that post-hoc analyses revealed that the significant limb by group interaction stemmed from between-limb differences within the respective group ($p < 0.05$). No significant interactions or main effects of limb or group were identified for intramuscular fat. In the case of significant interactions, main effects were not interpreted.

	Muscle	Involved ACLR	Uninvolved ACLR	Involved Control	Uninvolved Control	t	p value
Normalized Volume (mm^3/kg)	VL	**8875 ± 1651**	**9576 ± 1530**	9428 ± 1888	9342 ± 2107	-3.93	<0.001*
	VI	**7288 ± 1179**	**8003 ± 1092**	7309 ± 1565	7258 ± 1588	-4.10	<0.001*
	VM	**6416 ± 1250**	**7057 ± 1321**	6452 ± 1642	6434 ± 1751	-4.83	<0.001*
Fat-cleared Volume (mm^3/kg)	VL	**8729 ± 1615**	**9398 ± 1521**	9271 ± 1946	9180 ± 2092	-4.00	<0.001*
	VI	**7174 ± 1170**	**7876 ± 1078**	7199 ± 1589	7150 ± 1603	-3.93	<0.001*
	VM	**6308 ± 1235**	**6945 ± 1307**	6365 ± 1654	6339 ± 1745	-4.94	<0.001*
Intramuscular Fat (%)	VL	1.64 ± 1.26	1.90 ± 1.12	1.85 ± 1.43	1.81 ± 1.25	-0.62	0.536
	VI	1.60 ± 1.00	1.59 ± 1.03	1.63 ± 1.09	1.62 ± 1.08	-0.00	0.999
	VM	1.69 ± 0.87	1.60 ± 0.98	1.48 ± 0.91	1.58 ± 0.81	0.81	0.420

Table 13. Individual Vasti Muscle Volume and Intramuscular Fat. Data are reported as mean ± standard deviation. t statistics and p values reported are results of the limb by group interaction from the linear mixed effects model, with * denoting a statistically interaction at the level of p<0.05. Bold text indicates that post-hoc analyses revealed that the significant limb by group interaction stemmed from between-limb differences within the group (p < 0.05). In the case of significant interactions, main effects were not interpreted. No significant interactions or main effects of limb or group were identified for intramuscular fat.

5.4.3 Regional Comparison of Intramuscular Fat

Proximal, central, and distal intramuscular fat content is summarized in Table 14. When exploring if the amount of fat by region differed between groups and limbs, no significant group by limb interactions were identified. However, there was a significant main effect of region for the VL indicating that the proximal region had an 0.54 % greater fat fraction, independent of limb and group (p < 0.001). There was also a significant main effect of region for the VI and VM indicating that the distal region had a 0.93% and 0.36% greater fat fraction in the VI and VM, respectively, independent of limb and group (p < 0.001). Superficial and deep intramuscular fat content is

summarized in Table 15. No significant group by limb interactions were identified for any ROI. However, there was a significant main effect of region for the VI indicating that the superficial region had a 0.23 % greater fat fraction, independent of limb and group (p < 0.001).

Muscle	Region	Involved ACLR	Uninvolved ACLR	Involved Control	Uninvolved Control	t	p value
VL	Proximal$^{\alpha}$	2.24 ± 0.88	2.21 ± 1.05	2.27 ± 1.31	2.18 ± 1.09	-0.89	0.377
	Central	1.42 ± 1.40	1.80 ± 1.16	1.80 ± 1.48	1.70 ± 1.33		
	Distal	1.56 ± 1.53	1.80 ± 1.29	1.71 ± 1.61	1.83 ± 1.58		
VI	Proximal	1.64 ± 1.16	1.47 ± 1.16	1.64 ± 1.14	1.51 ± 1.03	0.47	0.641
	Central	1.24 ± 1.06	1.37 ± 0.95	1.48 ± 1.06	1.43 ± 1.09		
	Distal$^{\alpha}$	2.39 ± 1.49	2.25 ± 1.41	2.15 ± 1.35	2.45 ± 1.56		
VM	Proximal	1.57 ± 1.22	1.60 ± 1.03	1.52 ± 1.26	1.66 ± 0.93	0.96	0.337
	Central	1.41 ± 0.81	1.37 ± 0.89	1.45 ± 1.07	1.40 ± 0.91		
	Distal$^{\alpha}$	1.97 ± 1.04	1.81 ± 1.13	1.54 ± 0.74	1.75 ± 0.82		

Table 14. Regional Comparisons in proximo-distal direction of Intramuscular Fat (%) by Group and Limb. Data are reported as mean ± standard deviation. t statistics and p values reported are results of the limb by group interaction from the linear mixed effects model. No significant group by limb interactions were determined (p > 0.05). $^{\alpha}$ denotes a significant main effect of region that had increased intramuscular fat relative to the other regions. VL indicates vastus lateralis; VI, vastus intermedius; VM, vastus medialis.

Muscle	Region	Involved ACLR	Uninvolved ACLR	Involved Control	Uninvolved Control	t	p value
VL	Superficial	1.53 ± 1.26	1.80 ± 1.07	1.77 ± 1.39	1.69 ± 1.32	-1.07	0.286
	Deep	1.80 ± 1.23	2.04 ± 1.15	1.98 ± 1.44	1.97 ± 1.19		
VI	Superficial$^{\alpha}$	1.67 ± 1.08	1.74 ± 1.07	1.71 ± 1.21	1.74 ± 1.11	0.03	0.975
	Deep	1.52 ± 0.94	1.41 ± 1.01	1.54 ± 0.99	1.49 ± 1.07		
VM	Superficial	1.73 ± 0.92	1.66 ± 1.04	1.55 ± 0.92	1.65 ± 0.82	1.35	0.180
	Deep	1.65 ± 0.85	1.54 ± 0.92	1.41 ± 0.89	1.51 ± 0.84		

Table 15. Regional Comparisons by Depth of Intramuscular Fat (%) by Group and Limb. Data are reported as mean ± standard deviation. t statistics and p values reported are results of the limb by group interaction from the linear mixed effects model. No significant group by limb interactions were determined (p > 0.05). $^{\alpha}$ denotes a significant main effect of region that had increased intramuscular fat relative to the other regions. VL indicates vastus lateralis; VI, vastus intermedius; VM, vastus medialis.

5.4.4 Regional Variation of Intramuscular Fat

When exploring if the amount of fat varied by region within a limb (region by group by limb interaction) no significant interactions were identified (p > 0.05). Figure 19 illustrates regional distributions for each vasti muscle.
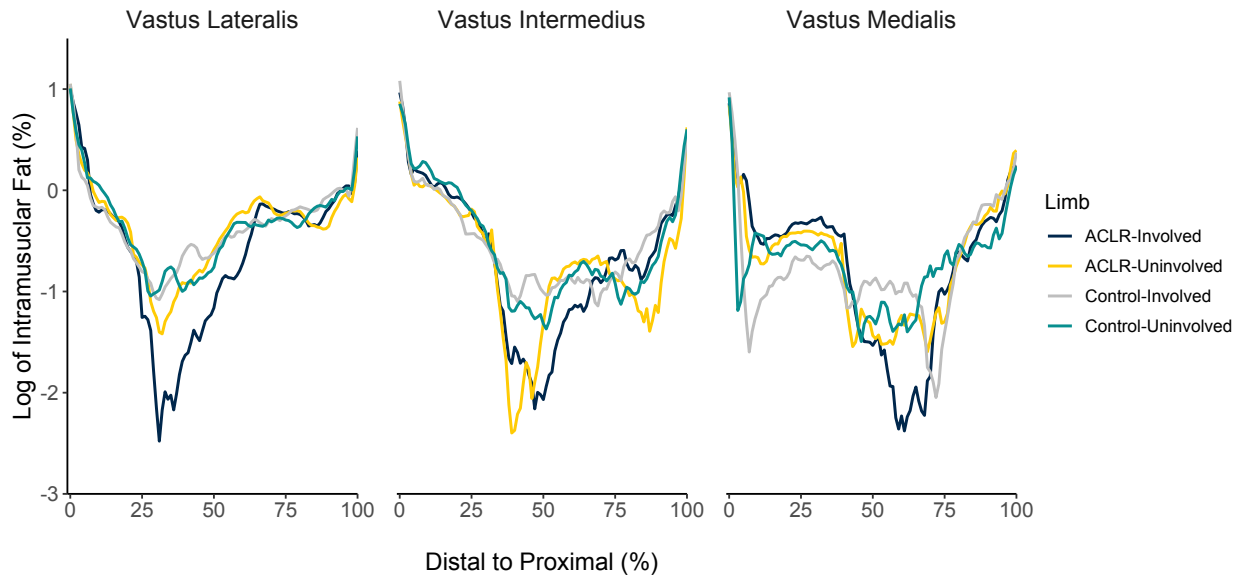
Figure 19. Log Transform of Regional Distributions of Intramuscular Fat for Individual Vasti Muscles by limb. Left, middle, and right panels demonstrate vastus lateralis, vastus intermedius, and vastus medialis, respectively

5.4.5 $T_{1\rho}/T_2$ Relaxation Times in Combined Weight-Bearing ROIs

$T_{1\rho}$ and T_2 relaxation times are summarized in Tables 16 and 17, respectively. There were no significant group by limb interactions for $T_{1\rho}$ in any combined weight bearing ROI (p = 0.257 – 0.981). There was a significant group by limb interaction for T_2 in the combined weight bearing ROIs in the lateral compartments (femur, p = 0.021; tibia, p = 0.038) but not the medial compartments (p = 0.560 – 0.870). Post hoc analyses indicated that the significant interaction for T_2 in the lateral femur stemmed from significant between limb differences in the Control group (p = 0.040, Cohens d = 0.610, 95% CI [0.0016, 1.22]) but no significant between-limb differences were identified in the tibia for both groups (p = 0.073 – 0.240).

When investigating $T_{1\rho}$ relaxation times in sub-ROIs, there were no significant group by limb interactions detected in the medial tibia (p = 0.062 – 0.412), lateral tibia (p = 0.259 – 0.845),

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or medial femur (p = 0.069 - 0.439). However, there was a significant group by limb interaction detected in the cLF-a (p = 0.035). Post hoc analyses revealed that the significant interaction stemmed from between limb differences in the Control group (p = 0.041, Cohen's d = 0.607, 95% CI [-0.001, 1.22]). No significant group by limb interactions were identified in the cLF-c or cLF-p (p = 0.286 – 0.782). There were also several significant main effects of limb and group for various sub-ROI's for $T_{1\rho}$ relaxation times. There was a significant main effect of limb in the cMT-a that indicated the uninvolved limbs had 9.8 ms longer $T_{1\rho}$ relaxation times than the involved limbs (p = 0.008). There was also a significant main effect of limb in the cMF-c, cMF-p, and the cLF-p that indicated the involved limbs had 6.6-8.8 ms longer $T_{1\rho}$ relaxation times than the uninvolved limbs (p = 0.009 – 0.045). There was also significant main effect of group in the cMT-a that indicated the Control group had 8.8 ms longer $T_{1\rho}$ relaxation times than the ACLR group, independent of limb (p = 0.018).

When investigating T_2 relaxation times in sub-ROIs, there were no significant group by limb interactions detected in the medial tibia (p = 0.204 – 0.853), lateral tibia (p = 0.148 – 0.274), or medial femur (p = 0.105 - 0.676). However, there was a significant group by limb interaction detected in the cLF-a (p = 0.029) and cLF-c (p = 0.029). Post hoc analyses revealed that the significant interaction stemmed from between limb differences in the Control group for the cLF-a (p = 0.011, Cohens d = 0.77, 95% CI [0.14, 1.39]) , but non-significant between-limb differences were identified for the cLF-c in both groups (ACLR, p = 0.156; Control, p = 0.089). No significant group by limb interactions were identified in the cLF-p (p = 0.326). There were also several significant main effects of limb and group for various sub-ROI's for T_2 relaxation times. There were significant main effects of limb for the cLT-p and cMF-a that indicated the uninvolved limbs had 3.8-4.1 shorter relaxation times relative to the involved limbs, independent of group (p =

0.011-0.002). There were also significant main effects of group for the cLF-p and cLT-p that indicated the Control group had 3.2 ms longer relaxation times in the cLF-p (p = 0.046) but 3.9 ms shorter relaxation times in the cLT-p (p = 0.014).

5.4.6 $T_{1\rho}/T_2$ Relaxation Times in Whole ROI's, Patella, Trochlea, and Outside Anterior and Posterior Meniscus Horns (Anterior/Posterior Femur)

There were significant group by limb interactions for $T_{1\rho}$ identified for the whole medial femur (p = 0.038) and medial patella (p = 0.047). Post hoc analyses revealed the significant interactions stemmed from between limb differences in the ACLR group, where the involved limb had 3.3 and 8.9 ms longer relaxation times than the uninvolved limb in the medial femur (p = 0.034, Cohens d = 0.632, 95% CI [0.02, 1.24]) and patella (p = 0.002, Cohens d = 0.96, 95% CI [0.31, 1.61]), respectively. No significant group by limb interactions were identified for $T_{1\rho}$ relaxation times in the lateral femur or patella, aMF, aLF, pMF, pLF, or the trochlea (p = 0.067 – 0.837). There were also several significant main effects of limb and group in various sub-ROI's for $T_{1\rho}$. A significant main effect of limb was determined for the trochlea and lateral patella that indicated the involved limbs had 5.2 and 7.8 ms longer $T_{1\rho}$ relaxation times than the uninvolved limbs in the trochlea (p = 0.021) and lateral patella (p = 0.007). A significant main effect of group was determined for the trochlea that indicated the ACLR group had 4.9 ms longer $T_{1\rho}$ relaxation times than the Control group (p = 0.043).

There was a significant group by limb interaction for T_2 identified for the aMF (p = 0.037) and pMF (p = 0.010). Post hoc analyses revealed the significant interactions stemmed from between limb differences in the ACLR group, where the involved limb had 7.7 ms and 3.4 ms longer relaxation times than the uninvolved limb in the aMF (p = 0.008, Cohens d = 0.89, 95% CI [0.19, 1.58]) and pMF (p < 0.001, Cohens d = 1.36, 95% CI [0.66, 2.07]), respectively. No

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significant group by limb interactions were identified for T_2 relaxation times in the medial or lateral femur, medial or lateral patella, aLF, pLF, or trochlea ($p = 0.071 – 0.510$). There were also several significant main effects of limb and group for various sub-ROI's for T_2 relaxation times. A significant main effect of limb was determined for the aLF, pLF, and medial and lateral femur that indicated the involved limbs had 1.7-3.1 ms longer T_2 relaxation times than the uninvolved limbs ($p = 0.001$-0.007). A significant main effect of group was determined for the pLF that indicated the ACLR group had 3.0 ms longer T_2 relaxation times than the Control group ($p = 0.039$).

5.4.7 Associations between Intramuscular Fat and Fat-Cleared Muscle Volume with Strength in the ACLR-involved and Control-involved limbs

In the ACLR-involved limb, covariates explained 29.5% of variance in strength outcomes ($F[2,21] = 4.38$, $p = 0.026$), where males and lower BMI's had higher strength (sex: $t = -2.08$, $p = 0.050$; BMI: $t = -2.65$, $p = 0.015$). Vasti intramuscular fat did not help to explain additional variance in the model ($\Delta F(1,20) = 3.13$, $\Delta p = 0.092$), nor did fat-cleared muscle volume ($\Delta F(1,20) = 2.34$, $\Delta p = 0.142$). In the Control-involved limb, covariates explained 31.1% of variance in strength outcomes ($F[2,21] = 4.74$, $p = 0.020$), where males had increased strength (sex: $t = -2.86$, $p = 0.009$; BMI: $t = -1.54$, $p = 0.140$). Vasti intramuscular fat did not help to explain additional variance in the model ($\Delta F(1,20) = 1.61$, $\Delta p = 0.219$), nor did fat-cleared muscle volume ($\Delta F(1,20) = 2.93$, $\Delta p = 0.102$).

5.4.8 Associations between Intramuscular Fat, Fat-Cleared Muscle Volume, and Strength, with Cartilage Composition in the ACLR-involved limb

Covariates (i.e., BMI and sex) explained 4.2-5.5% of the variance in $T_{1\rho}$ relaxation times for the medial (F[2,21] = 0.61, p = 0.552) and lateral (F[2,21] = 0.46, p = 0.637) patella. After accounting for covariates, vasti intramuscular fat explained an additional 24.5-25.6% of the variance in the medial (Δ F(1,20) = 7.43, Δ p = 0.013) and lateral (Δ F(1,20) = 6.89, Δ p = 0.016) patella (Figure 20). Less intramuscular fat was related to longer relaxation times (medial: ß = -.53, t = -2.73; lateral: ß = -.52, t = -2.63). Strength and fat-cleared muscle volume were not associated with $T_{1\rho}$ in the medial or lateral patella (p > 0.05).

No significant associations were identified between total vasti intramuscular, fat-cleared muscle volume, or strength with $T_{1\rho}$ in the lateral femur (p > 0.05). However, covariates explained 41.2 % of the variance in $T_{1\rho}$ relaxation time in the medial femur (F[2,21] = 7.35, p = 0.004), where males had significantly longer $T_{1\rho}$ relaxation times (t = -3.83, p = 0.001). Vasti intramuscular fat did not help to explain additional variance in the model (Δ F(1,20) = 0.56, Δ p = 0.463), nor did strength (Δ F(1,20) = 0.01, Δ p = 0.944) or fat-cleared muscle volume (Δ F(1,20) = 3.29, Δ p = 0.085).

No significant associations were identified between total vasti intramuscular, fat-cleared muscle volume, or strength with $T_{1\rho}$ in the lateral tibia (p > 0.05). However, covariates explained 28.5 % of the variance in $T_{1\rho}$ relaxation time in the medial tibia (F[2,21] = 4.19, p = 0.029), where males had significantly longer $T_{1\rho}$ relaxation times (t = -2.46, p = 0.023). Vasti intramuscular fat did not help to explain additional variance in the model (Δ F[1,20] = 0.34, Δ p = 0.565), nor did strength (Δ F[1,20] = 1.42, Δ p = 0.247) or fat-cleared muscle volume (Δ F[1,20] = 0.02, Δ p =

0.897). No significant associations were identified for total vasti intramuscular, fat-cleared muscle volume, or strength with T_2 relaxation times in any whole ROI (femur or patella) or combined weight-bearing ROI (femur, tibia).

Given the significant association between total vasti intramuscular fat and $T_{1\rho}$ in the medial and lateral patella, we investigated if intramuscular fat of individual vasti muscles had similar associations in the medial and lateral patella. After accounting for covariates, intramuscular fat of the VL, VI, and VM helped to explain 16.4%, 28.8%, and 24.1% of additional variance in the medial patella, respectively. However, only the addition of intramuscular fat of the VI and VM was significant (VL: $\Delta F[1,20] = 4.19$, $\Delta p = 0.054$; VI: $\Delta F[1,20] = 8.79$, $\Delta p = 0.008$; VM: $\Delta F[1,20] = 6.84$, $\Delta p = 0.017$). After accounting for covariates, intramuscular fat of the VL, VI, and VM helped to explain 30.6%, 21.0%, and 10.8% of additional variance in the lateral patella, respectively. However, only the addition of intramuscular fat of the VL and VI were significant (VL: $\Delta F[1,20] = 9.38$, $\Delta p = 0.006$; VI: $\Delta F[1,20] = 5.60$, $\Delta p = 0.028$; VM: $\Delta F[1,20] = 2.55$, $\Delta p = 0.126$). In all significant cases, less intramuscular fat was associated with longer $T_{1\rho}$ relaxation times in the medial or lateral patella.
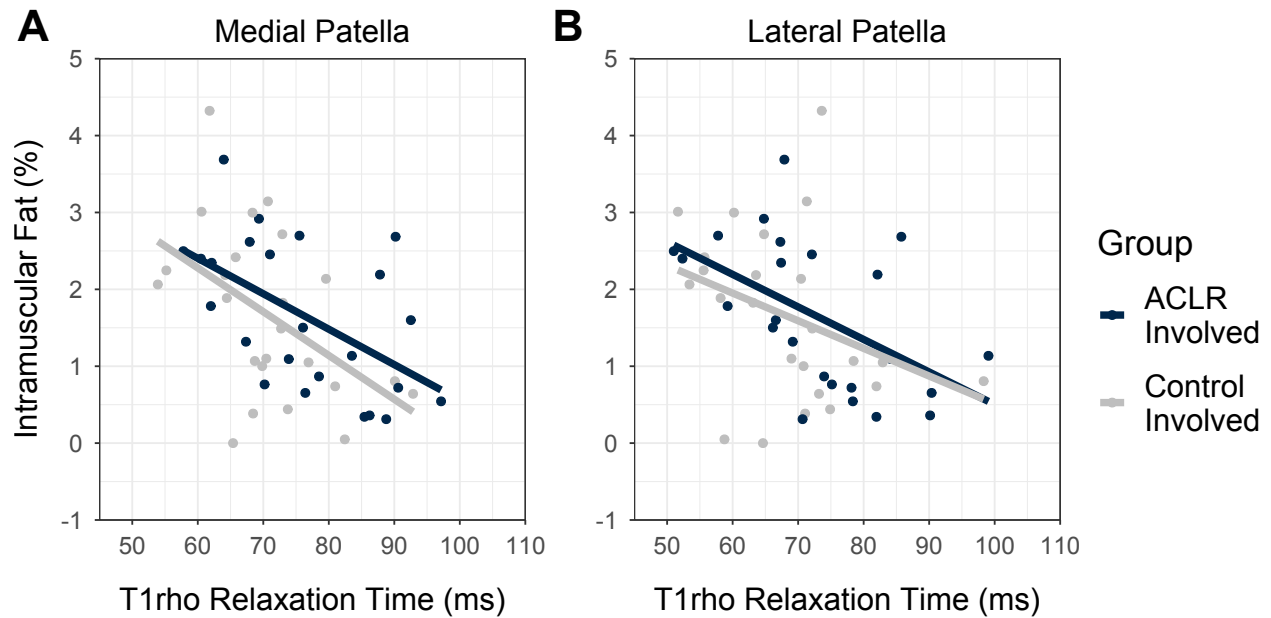
Figure 20. Associations Between Intramuscular Fat (%) and T1rho in the Medial (A) and Lateral (B) Patella for the ACLR-Involved and Control-Involved Limbs. After accounting for covariates of BMI and sex, vasti intramuscular fat accounted for on average 25% of variance in T1rho relaxation times in the medial and lateral patella in the ACLR-involved limb. In the Control-involved limb, vasti intramuscular fat accounted for on average 25% of variance in T1rho relaxation times in the medial patella but not the lateral patella.

5.5 Discussion

The objectives of our study were to determine if muscle size, composition, and strength were predictive of worse cartilage outcomes, as measured by increased $T_{1\rho}/T_2$ relaxation times, and to determine if muscle size and composition are associated with strength. Additionally, we aimed to comprehensively evaluate the intramuscular fat of the vasti quadriceps muscles and determine if early compositional changes to the cartilage matrix (i.e., proteoglycan depletion, type II collagen disorganization) were present in the ACLR limbs compared to contralateral and control limbs. We hypothesized that smaller, weaker muscles with increased intramuscular fat would be associated with longer $T_{1\rho}/T_2$ relaxation times, indicating degenerative cartilage changes. The primary results of our study indicated that those with ACLR exhibited smaller quadriceps muscle volumes

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compared to contralateral and control limbs, and poorer cartilage compositional metrics in various ROIs – evidence by longer $T_{1\rho}/T_2$ relaxation times. We also observed that vasti intramuscular fat was negatively associated with $T_{1\rho}$ relaxation times in the ACLR and Control groups, indicating that lower levels of fat were linked with poorer proteoglycan concentrations. In addition, quadriceps intramuscular fat content and quadriceps strength outcomes did not differ across limbs or groups. This level of strength recovery contrasts with many other reports[24,25] and signifies a promising outcome as it emphasizes the potential of achieving adequate strength recovery post-ACLR. Overall, our data suggest those with ACLR demonstrate quadriceps muscle atrophy and early markers of cartilage degeneration in the involved limb despite achieving relatively similar quadriceps strength and having similar levels of intramuscular fat compared to contralateral limbs and uninjured controls.

Given the disruptive capabilities of intramuscular fat on force-producing and force-transmitting properties of muscle and the commonly reported strength deficits following ACLR we hypothesized that poorer quadriceps muscle properties (i.e., atrophy, increased intramuscular fat, strength deficits) would be linked with early degenerative changes in cartilage composition in ACLR limbs. In contradiction to our hypotheses, muscle size, strength, and intramuscular fat were largely unassociated with $T_{1\rho}/T_2$ relaxation times in both the ACLR and Control groups with the exception of patellar cartilage. While we detected that about 25% of variance in medial patella $T_{1\rho}$ relaxation times could be explained by total vasti intramuscular fat, this association was similar across groups and limbs and showed a negative relationship between $T_{1\rho}$ and intramuscular fat. Further, intramuscular fat was similarly associated with lateral patella $T_{1\rho}$ relaxation times in the ACLR group, but this was not observed in the Control group. These findings are interesting and contradict our original hypotheses as they suggest those with less intramuscular muscle content

exhibit poorer MRI-markers of cartilage composition, independent of ACLR. In this scenario, it remains possible that quadriceps intramuscular fat is an indicator of poor muscle quality but is not a direct contributor to cartilage degeneration or accumulates at later stages in the PTOA disease process. While no study has directly investigated quadriceps intramuscular fat with $T_{1\rho}/T_2$ relaxation times of knee cartilage following ACLR, others have shown that a reduction in VM intramuscular fat infiltration was beneficial to maintain knee cartilage volume in the medial patella in high BMI (> 27 kg/m^2) adults.[97] For instance, it has also been shown that patients with idiopathic knee osteoarthritis have an increase in VM[290] and total quadriceps intramuscular fat,[98] and that VM intramuscular fat is related to reduced quadriceps strength.[291] Importantly, the increases in quadriceps intramuscular fat were in patients with knee osteoarthritis who also exhibited strength deficits. As such, it is possible that fatty infiltration occurs later in the PTOA disease process beyond the three year time point investigated in this study and is more likely to affect patients who present with strength deficits and decreased activity levels after ACLR.

We also observed that quadriceps muscle volume and intramuscular fat were not associated with quadriceps strength in the ACLR and Control groups. This is particularly interesting as the total vasti volume and each individual vasti muscle volume in the ACLR-involved limb was 7-9% smaller than the uninvolved limb. It is well known that following ACLR many patients experience persistent strength deficits, which often co-exists with muscle atrophy.[33] However, it has been shown that muscle size doesn't fully explain muscle strength. Thomas et al.[33] found that quadriceps cross-sectional area only explained 30% of isometric quadriceps strength deficits in the acute stages (7 months) following ACLR, while Arangio et al.[292] found no correlation between thigh circumference and strength in the ACLR-involved limb 4 years following ACLR. While there are limitations of the assessments used to measure cross-sectional area and thigh circumference as

they can include other elements of muscle composition like non-contractile elements and subcutaneous fat, these results provide evidence that muscle size only helps to explain some of the variance in muscle strength. Nevertheless, the findings of the present study add to this body of work thereby indicating that persistent atrophy can manifest even in the absence of strength deficits. The clinical meaningfulness of muscle atrophy, while able to achieve adequate strength, requires further investigation. However, it is important to recognize that muscle strength is an isolated assessment of muscle function that does not represent how the muscle is utilized during activities of daily living and of young, athletic populations,[293] which is the primary population affected by ACL injuries. To this point, muscle function has been largely and historically categorized as simplified measures of muscle strength, and size. However, these simplified muscle properties do not encompass the intrinsic properties of muscle that give rise to its contractile ability, including its composition (e.g., fat and fibrotic tissue, fiber types, inflammation), neural activation, or muscle architecture. The ACLR cohort included in this study was far removed from surgery (Table 10), able to recover bilateral quadriceps strength and were physically active. As such, it remains plausible that our cohort doesn't represent the majority of those following ACLR who exhibit lingering strength deficits long after completing rehabilitation, which may still be influenced by the accumulation of intramuscular fat. It is also possible that there are changes in intramuscular fat earlier after surgery that may affect muscle recovery. Further research is warranted to explore longitudinal effects of muscle composition and how they impact muscle recovery in the acute stages following ACLR.

We anticipated that there would be an increase in intramuscular fat that would differ regionally and in its distribution in patients with a history of ACLR. However, we discovered that there were no global or regional differences, or variability in the distribution of intramuscular fat

relative to the uninjured limb or to healthy controls. There are multiple methods to measure fat utilized in the literature. Others have shown global elevations of intramuscular fat in the ACLR limb at chronic time-points post-ACLR measured by hypoechoic signaling from ultrasound (unpublished findings presented at ACL Retreat 2019).[294] While in other populations, fat has been characterized by fatty streaks or localized deposits that can vary throughout muscle regions.[284] While this is the first study to utilize MRI to comprehensively measure intramuscular fat following ACLR, we demonstrate through comprehensive measurement techniques (MRI on every slice) that intramuscular fat does not differ from that of healthy controls and uninjured limbs at chronic time points following ACLR. However, performing longitudinal study designs across more diverse subjects (e.g., range of activity levels, BMI's, quadriceps function) are needed to substantiate this finding and to determine if intramuscular fat interferes with muscle recovery earlier after ACLR.

We also identified a variety of between-limb and group differences in the ACLR and Control groups for $T_{1\rho}/T_2$ relaxation times. Interestingly, the primary differences detected in the Control group were isolated to the combined weight bearing ROI's (lateral femur, and medial/lateral tibia), while the primary differences detected in the ACLR group were identified in the whole medial femur, patella, trochlea, aMF, pMF, and pLF (including significant interactions and significant group effects). As expected, in all significant interactions or group effects for the ACLR group, the involved limb had a longer $T_{1\rho}/T_2$ relaxation time than the uninvolved limb or the ACLR group had longer $T_{1\rho}/T_2$ relaxation times than the Control group thereby indicating, more degenerative changes in cartilage composition in these ROIs. However, there were also between-limb differences in the Control group detected for the combined weight-bearing femur and cLF-a and significant group effects in the cLF-p, cMT-a, and cLT-p, which had both shorter and longer relaxation times relative to the ACLR group. As the primary between-limb and group differences

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of our Control group primarily resided in the lateral compartments within the weight-bearing ROIs, it is possible that the medial compartments are more susceptible to degenerative changes following ACLR and the lateral compartments may be more resilient to load and changes in tissue composition that permit a wider range of normative $T_{1\rho}/T_2$ relaxation times. To this point, changes in the medial compartments are consistent with others who have shown that normal kinematics on the lateral compartment are restored following ACLR, but not the medial.[295] Further, the ACLR group exhibited large effect sizes for the between-limb differences in the medial patella (Cohen's d = .96), aMF (Cohen's d = .89), and pMF (Cohen's d = 1.36), indicating substantial and clinically meaningful distinctions between-limbs. In contrast to this, the Control group demonstrated medium effect sizes for all of the between-limb differences, suggesting a comparatively smaller magnitude of variation between-limbs. This is an important consideration when interpreting the findings of these data and for future directions to set thresholds for identifying clinically meaningful significant between-limb differences in $T_{1\rho}/T_2$ relaxation times as measurements of components in the cartilage matrix. Given the differences in effect sizes, we direct the focus of the discussion regarding $T_{1\rho}/T_2$ relaxation times to the ACLR group.

Some of our findings in the ACLR group are similar to others who have reported elevations in the ACLR-involved limb for $T_{1\rho}/T_2$ in the medial femur (global and various weight-bearing ROIs) from 6 months to five years post-ACLR.[164,273,296-298] Interestingly, the posterior ROI's of the medial and lateral femur were both affected by ACLR. While this ROI is less likely to be considered weight-bearing, Li et al.[299] has shown these ROIs are loaded throughout the stance phases of gait, with increased contact as a knee undergoes more knee flexion in obese and non-obese patients.[299] In addition, Bolcos et al.[300] found that posterior regions had disproportionately increased maximal principal stresses that were associated with longer T_2 relaxation times (r = 0.71,

p < 0.001) in ACLR patients. Notably, much work has revolved around understanding joint loading with $T_{1\rho}/T_2$ relaxation times and have found both under- and over-loading strategies are associated with elevated $T_{1\rho}/T_2$ relaxation times.[301] In Chapter 4, we found that this ACLR-patient cohort walked with an underloading strategy. As such, we speculate that these posterior areas may be underloaded at chronic time points following ACLR and may be associated with a decrease loading which may be related to the longer T2 relaxation times found in the current study. Although the patellofemoral joint (patella, trochlea) has been less studied in relation to the tibiofemoral joint following ACLR, there have been elevations in $T_{1\rho}/T_2$ relaxation times demonstrated in these ROIs.[302] To this point, many of the studies investigating $T_{1\rho}/T_2$ relaxation times following ACLR do not consider patella cartilage and/or included a variety of graft types.[297,303,304] The included study only consisted of patients who received a patella-tendon graft type. As such, it is possible that the significance changes we found in the medial patella and trochlea are related to graft type. Further research is needed across graft types to understand if graft harvest influence post-operative outcomes and precipitate PTOA of the patellofemoral joint.

There are multiple limitations to consider while interpreting the findings of this study. First, this was a cross-sectional study design that did not capture longitudinal muscle or cartilage outcomes. As muscle and cartilage composition and quadriceps function are modifiable, understanding the time-course of changes rather than just at a single time point may help better understand the link between abnormal muscle properties, quadriceps function, and cartilage health post-ACLR. It is plausible the interrelationship between these factors differ across the post-operative rehabilitation time course wherein stronger associations are observed earlier in recovery as opposed to later time points, as in the current study. There are also multiple limitations to consider at both the muscle and cartilage level.

At the muscle level, it is important to recognize that we only explored intramuscular fat and did not explore intermuscular fat, that can encompass both intramuscular fat within a tissue and irregular fat deposits between muscle groups.[305] Similarly to intramuscular fat, intermuscular fat has been associated with a host of impairments including impaired mobility and muscle dysfunction.[305] Either type of fatty deposits (e.g., intramuscular and intermuscular) can alter mechanical properties of muscle tissue (e.g., stiffness) that disrupts how muscle fibers shape change and interact the aponeuroses to generate force during movement.[225] Given that intermuscular fat has not been fully characterized or explored following ACLR, future research would benefit from understanding if it is elevated following ACLR and determining its functional impact. Another compositional component that may interfere with contractile function in a similar manner to fat is the development of fibrotic tissue. Fibrotic tissue has been less studied following ACLR but there is early evidence to support expansions of the extracellular matrix through increases in collagen.[37] As such, this is a compositional component of muscle that was not captured by our mDixon MRI sequence but has the potential to influence muscle function. Understanding if there is a replacement of healthy contractile tissue with non-contractile elements like fibrotic tissue development is an important future direction for our field in characterizing muscle dysfunction following ACLR. Interestingly, our ACLR and Control groups had the same ratio of fat to muscle. However, it remains unknown if the ratio of fat to muscle changes by muscle size or over a range of athletic populations. Here, all participants had the same activity level at the time of testing that could be described as between recreational and competitive sports. This may help to explain why we didn't see variability in the quantity of intramuscular fat in the ACLR and Control cohorts as it has been shown that consistent physical activity, endurance, and resistance training can all decrease quadriceps intramuscular fat,[55-57] and help to preserve cartilage

volume.[58] However, since the ACLR vasti muscles had atrophy, the percent of fat may change as a function of size. Further research is needed to understand the role of intramuscular and intermuscular fat for muscle function and to understand how much intramuscular and intermuscular fat is normal based on activity level.

While $T_{1\rho}/T_2$ are useful measures of cartilage composition, there are many considerations to consider. $T_{1\rho}/T_2$ relaxation times are largely variable as a consequence of non-standardized methods that differ by MRI scanners, image sequences, and image processing techniques across research sites. To overcome this, we explored effect sizes and the amount of change compared to the other limbs. While our findings of 3-10 ms differences in relaxation times is comparable to other groups, it remains unknown the functional impact of these findings in particular given that we also identified between-limb differences in the Control group. Further research is needed to characterize healthy $T_{1\rho}/T_2$ relaxation times or relative between-limb differences in $T_{1\rho}/T_2$ that applies across research sites and scanners in order to understand the clinical impact of these findings. In addition, understanding how well degenerative changes identified through longer $T_{1\rho}/T_2$ relaxation times translate to structural and symptomatic PTOA is crucial to give clinical meaning to these imaging modalities and fully utilize their potential in identifying early indicators of PTOA through changes in cartilage composition. Furthermore, we utilized a 3T MRI scanner for our current investigation, but it is likely a magnet with higher spatiotemporal and spectral resolution may help better capture structural and compositional changes. In addition, laminar analyses are an important next step to further characterize the extent of degeneration by cartilage depth level. This is an important future direction to overcome some of the current limitations that constrain our interpretation and clinical meaning of how changes in $T_{1\rho}/T_2$ are related to structural cartilage abnormalities. It is also important to recognize that cartilage is a dynamic and adaptable

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tissue that responds to load. We only evaluated cartilage $T_{1\rho}/T_2$ at rest. Alterations in cartilage composition strongly impact the tissues' ability to withstand weightbearing and assessing how the tissue acutely responds to mechanical stimuli is theorized to help understand early disruption in cartilage mechanical properties. Future work should consider evaluating how cartilage structure, and composition changes in response to acute loading paradigms in those with ACLR to improve our understanding of early OA-related cartilage alterations. Given that there is limited work investigating the relationships between intramuscular fat and cartilage health, we powered our study off of extramusuclar fat and Kellgren-Lawrence grading scores. As such, it is possible that the current study is underpowered and would benefit from large sample sizes. Lastly, cartilage composition can be influenced by concomitant injuries like damage to the meniscus, bone bruises, cartilage lesions and by lower extremity alignment which were not captured in the current study but have been shown to contribute to elevated $T_{1\rho}/T_2$ relaxation times.[303]

This study highlights that intramuscular fat is primarily associated with medial and lateral patella $T_{1\rho}$ relaxation times but there is no difference in these associations between ACLR and Control groups. Despite experiencing reduced quadriceps muscle size following ACLR, patients also showed similarities in intramuscular fat and strength compared to healthy controls. Further, both ACLR and Controls exhibited between limb differences in $T_{1\rho}/T_2$ relaxation times in various ROIs. This work is clinically significant as it combines quantitative metrics of muscle and joint health, which when examined in concert help us more directly determine the effect of muscle dysfunction on joint disease following ACLR. Future work is needed to establish normative thresholds of $T_{1\rho}/T_2$ by ROI and to understand how they are related to muscle and cartilage properties that precipitate PTOA.

Location/ Compartment	Region	ACLR Involved	ACLR Uninvolved	Control Involved	Control Uninvolved	t	p value
Femur — Medial	Whole	**69.7 ± 8.9**	**66.3 ± 7.9**	67.1 ± 4.9	68.4 ± 5.1	2.14	0.038*
	Anterior	73.3 ± 17.2	81.3 ± 16.4	73.7 ± 14.3	74.6 ± 9.9	-1.09	0.281
	WB	75.7 ± 13.6	70.3 ± 9.3	74.2 ± 7.3	73.2 ± 9.1	1.14	0.257
	c-Anterior	71.0 ± 16.3	77.6 ± 13.8	77.8 ± 14.5	79.8 ± 17.4	-0.78	0.439
	c-Central$^\beta$	76.0 ± 16.4	69.0 ± 11.2	73.3 ± 9.1	75.2 ± 14.2	1.84	0.069
	c-Posterior$^\beta$	79.1 ± 14.7	70.3 ± 10.4	75.4 ± 10.9	71.9 ± 9.91	1.15	0.254
	Posterior	64.7 ± 10.0	62.2 ± 8.2	60.8 ± 7.1	64.2 ± 6.6	1.88	0.067
Femur — Lateral	Whole	69.1 ± 10.0	67.6 ± 4.8	66.7 ± 6.1	64.6 ± 5.9	-0.21	0.837
	Anterior	74.3 ± 9.3	70.1 ± 8.8	70.6 ± 11.8	64.6 ± 6.2	-0.48	0.631
	WB	72.6 ± 16.1	70.8 ± 6.1	71.9 ± 8.6	70.0 ± 7.3	-0.02	0.981
	c-Anterior	64.2 ± 15.6	67.2 ± 9.3	**67.7 ± 12.7**	**61.2 ± 9.9**	-2.17	0.035*
	c-Central	72.7 ± 18.1	72.6 ± 9.8	75.0 ± 11.1	73.4 ± 11.0	-0.28	0.782
	c-Posterior$^\beta$	78.0 ± 18.6	71.3 ± 6.4	72.2 ± 7.9	70.0 ± 6.8	1.08	0.286
	Posterior	60.3 ± 11.6	61.5 ± 8.0	61.1 ± 7.4	60.3 ± 8.2	-0.65	0.516
Tibia — Medial	WB	69.0 ± 11.1	66.5 ± 8.7	69.0 ± 7.6	68.8 ± 7.0	0.68	0.500
	c-Anterior$^{\alpha\gamma}$	62.6 ± 14.8	72.5 ± 14.5	71.7 ± 11.4	71.8 ± 10.5	-1.89	0.062
	c-Central	74.4 ± 14.3	71.8 ± 12.3	73.9 ± 9.1	75.9 ± 8.8	1.01	0.315
	c-Posterior	64.7 ± 13.0	59.3 ± 8.2	63.8 ± 9.3	61.7 ± 8.3	0.82	0.412
Tibia — Lateral	WB	67.1 ± 11.6	64.8 ± 7.3	66.5 ± 5.5	65.0 ± 6.6	0.23	0.822
	c-Anterior	66.1 ± 12.9	71.6 ± 10.6	71.1 ± 14.0	70.9 ± 10.9	-1.14	0.259
	c-Central	68.2 ± 12.6	64.3 ± 7.8	67.9 ± 6.7	66.0 ± 7.75	0.58	0.566
	c-Posterior	67.2 ± 13.0	64.2 ± 9.0	63.4 ± 6.1	61.2 ± 7.2	0.20	0.845
Patella — Medial	Whole	**76.4 ± 11.5**	**67.5 ± 9.1**	71.0 ± 9.6	69.7 ± 6.6	2.02	0.047*
Patella — Lateral	Whole$^\beta$	73.0 ± 12.1	65.2 ± 7.3	68.2 ± 10.8	67.1 ± 7.8	1.65	0.103
Trochlea	Whole$^{\beta\delta}$	71.4 ± 9.3	66.2 ± 7.0	66.6 ± 8.9	65.4 ± 7.7	1.32	0.194

Table 16. $T_{1\rho}$ Relaxation Times in Whole and Sub-Regions of Interest (ROI). Data are reported in milliseconds and as mean ± standard deviation. t statistics and p values reported are the result of the limb by group interaction from the linear mixed effects model, with * denoting a statistically interaction at the level of $p<0.05$. Bold text indicates that post-hoc analyses revealed that the significant limb by group interaction stemmed from between-limb differences within the group ($p < 0.05$). $^\alpha$ or $^\beta$ indicates a significant main effect of limb where the involved limbs had shorter or longer relaxation times relative to the uninvolved limbs, respectively. $^\gamma$ or $^\delta$ indicates a significant main effect of group where the ACLR group had shorter or longer relaxation times relative to the Control group, respectively. WB indicates combined weight-bearing ROI; c-, central sub-ROI of the combined weight-bearing ROI.

Location/ Compartment		Region	ACLR Involved	ACLR Uninvolved	Control Involved	Control Uninvolved	t	p value
Femur	Medial	Whole$^\beta$	49.9 ± 5.1	48.2 ± 4.8	48.4 ± 2.9	48.0 ± 3.5	1.52	0.135
		Anterior	**58.4 ± 15.1**	**50.9 ± 6.9**	49.8 ± 5.12	50.8 ± 6.1	2.00	0.049*
		WB	50.6 ± 7.3	51.0 ± 5.9	51.4 ± 4.1	51.5 ± 4.5	-0.16	0.870
		c-Anterior$^\beta$	53.1 ± 7.9	49.0 ± 4.6	51.6 ± 5.2	50.3 ± 6.1	1.65	0.105
		c-Central	48.7 ± 10.3	49.4 ± 6.7	50.5 ± 6.3	52.1 ± 5.9	0.42	0.676
		c-Posterior	52.7 ± 7.1	54.1 ± 7.1	52.5 ± 4.6	51.7 ± 5.5	-1.35	0.184
		Posterior	**48.9 ± 4.5**	**45.5 ± 4.8**	45.8 ± 3.8	45.1 ± 3.4	2.68	0.010*
	Lateral	Whole$^\beta$	51.4 ± 4.9	49.6 ± 4.0	50.8 ± 2.7	49.6 ± 2.8	0.82	0.419
		Anterior$^\beta$	54.0 ± 4.3	51.3 ± 4.4	51.9 ± 3.0	50.6 ± 3.4	1.29	0.202
		WB	50.1 ± 6.6	51.3 ± 5.0	**53.7 ± 4.5**	**51.6 ± 5.1**	-2.39	0.021*
		c-Anterior	49.6 ± 9.6	50.7 ± 8.3	**50.8 ± 9.0**	**45.3 ± 8.4**	-2.26	0.029*
		c-Central	49.0 ± 8.2	50.9 ± 5.2	53.4 ± 5.5	51.2 ± 6.4	-2.25	0.029*
		c-Posterior$^\gamma$	52.0 ± 6.1	52.9 ± 6.3	55.3 ± 4.7	54.9 ± 4.9	-0.99	0.326
		Posterior$^{\beta\delta}$	49.5 ± 6.0	46.4 ± 6.3	46.5 ± 3.5	46.2 ± 3.2	1.85	0.071
Tibia	Medial	WB	39.4 ± 4.1	39.5 ± 3.4	39.8 ± 3.2	40.5 ± 3.1	0.59	0.560
		c-Anterior	40.8 ± 6.3	41.5 ± 5.0	43.2 ± 5.8	43.6 ± 5.2	-0.21	0.832
		c-Central	37.0 ± 4.8	38.1 ± 4.3	38.3 ± 5.1	39.1 ± 4.1	-0.19	0.853
		c-Posterior	42.7 ± 5.4	40.9 ± 4.2	41.4 ± 3.8	41.3 ± 4.1	1.29	0.204
	Lateral	WB	35.8 ± 4.3	36.5 ± 4.0	**36.9 ± 3.0**	**35.8 ± 3.2**	-2.14	0.038*
		c-Anterior	38.1 ± 4.8	40.8 ± 8.1	39.0 ± 5.9	39.3 ± 4.4	-1.11	0.274
		c-Central	31.7 ± 5.1	32.1 ± 4.4	32.5 ± 3.5	31.7 ± 4.2	-1.35	0.185
		c-Posterior$^{\beta\delta}$	48.7 ± 7.7	44.9 ± 5.5	44.8 ± 3.1	44.0 ± 4.5	1.47	0.148
Patella	Medial	Whole	44.9 ± 5.1	43.7 ± 3.4	45.0 ± 3.8	45.3 ± 4.8	1.19	0.240
	Lateral	Whole	44.0 ± 4.8	44.8 ± 4.4	44.9 ± 3.7	45.1 ± 3.3	-0.66	0.510
Trochlea		Whole	51.6 ± 4.2	51.0 ± 4.1	50.7 ± 4.0	50.7 ± 3.2	0.70	0.489

Table 17. T_2 Relaxation Times in Whole and Sub-Regions of Interest (ROI). Data are reported in milliseconds and as mean ± standard deviation. t statistics and p values reported is result of the limb by group interaction from the linear mixed effects model, with * denoting a statistically interaction at the level of $p<0.05$. Bold text indicates that post-hoc analyses revealed that the significant limb by group interaction stemmed from between-limb differences within the group ($p < 0.05$). $^\alpha$ or $^\beta$ indicates a significant main effect of limb where the involved limbs had shorter or longer relaxation times relative to the uninvolved limbs, respectively. $^\gamma$ or $^\delta$ indicates a significant main effect of group where the ACLR group had shorter or longer relaxation times relative to the Control group, respectively WB indicates combined weight-bearing ROI; c-, central sub-ROI of the combined weight-bearing ROI.

5.5.1   Supplementary Material

5.5.1.1 Associations Between Time Since Surgery with Muscle and Cartilage Properties in the ACLR-involved limb

Time since surgery was not associated with muscle volume ($p = 0.642$), intramuscular fat ($p = 0.671$), strength ($p = 0.195$), $T_{1\rho}$ in the medial femur ($p = 0.759$) and patella ($p = 0.832$), or T_2 in the medial ($p = 0.808$) and lateral ($p = 0.353$) posterior femur. However, there was a significant association between time since surgery and $T_{1\rho}$ relaxation times in the trochlea that indicated more time after surgery was associated with a 5.0 ms decrease in $T_{1\rho}$ relaxation times ($p = 0.044$).

5.5.1.2 Associations between Intramuscular Fat, Fat-Cleared Muscle Volume, and Strength, with Cartilage Composition in the Control-involved

No significant associations were identified between covariates, total vasti intramuscular, fat-cleared muscle volume, or strength with $T_{1\rho}$ in the lateral patella ($p > 0.05$). However, covariates explained 8.5% of the variance in $T_{1\rho}$ relaxation times in the medial patella ($F[2,21] = 0.97$, $p = 0.394$). After accounting for covariates, total vasti intramuscular fat explained an additional 25% of the variance in the medial patella ($\Delta F(1,20) = 7.50$, $\Delta p = 0.013$). Less intramuscular fat was related to longer relaxation times ($\beta = -.55$, $t = -2.74$). Strength and fat-cleared muscle volume were not associated with $T_{1\rho}$ in the medial patella ($p = 0.653 - 0.738$).

Covariates explained 26.3 % of the variance in $T_{1\rho}$ relaxation time in the lateral femur ($F[2,21] = 3.76$, $p = 0.040$), where males had significantly longer $T_{1\rho}$ relaxation times ($t = -2.47$, $p = 0.022$). Vasti intramuscular fat did not help to explain additional variance in the model ($\Delta F(1,20) = 3.46$, $\Delta p = 0.078$), nor did strength ($\Delta F(1,20) = 1.20$, $\Delta p = 0.287$) or fat-cleared muscle volume ($\Delta F(1,20) = 0.356$, $\Delta p = 0.557$). No significant associations were identified between covariates,

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total vasti intramuscular, fat-cleared muscle volume, or strength with $T_{1\rho}$ in the medial femur ($p >$ 0.05).

Covariates explained 6.8% of the variance in $T_{1\rho}$ relaxation time in the lateral tibia (F[2,21] = 0.77, p = 0.476). After accounting for covariates, total vasti intramuscular fat helped to explain an additional 17.4% of variance in the lateral tibia (Δ F(1,20) = 3.46, Δ p = 0.045). More intramuscular fat was related to longer relaxation times (ß = .46, t = 2.14). Strength and fat-cleared muscle volume were not associated with $T_{1\rho}$ in the lateral tibia (p =0.105 - 0.249). No significant associations were identified between covariates, total vasti intramuscular, fat-cleared muscle volume, or strength with $T_{1\rho}$ in the medial tibia ($p >$ 0.05).

No significant associations were identified for covariates, total vasti intramuscular, fat-cleared muscle volume, or strength with T_2 relaxation times in the medial or lateral femur or patella, or medial tibia ($p >$ 0.05). However, covariates explained 26.6% of the variance in T_2 relaxation time in the lateral tibia (F[2,21] = 3.80, p = 0.039), where males had shorter relaxation times than females (t = 2.35, p = 0.028). Vasti intramuscular fat did not help to explain additional variance in the lateral tibia (Δ F(1,20) = 0.17, Δ p = 0.682), nor did strength (Δ F(1,20) = 0.05, Δ p = 0.820) or fat-cleared muscle volume (Δ F(1,20) = 0.289, Δ p = 0.597).

Given the significant association between total vasti intramuscular fat and $T_{1\rho}$ in the medial patella and lateral tibia, we investigated if intramuscular fat of individual vasti muscles had similar associations. After accounting for covariates, intramuscular fat of the VL, VI, and VM helped to explain 25.5%, 24.0%, and 19.3% of additional variance in the medial patella, respectively. The addition of intramuscular fat of all vasti muscles was significant (VL: Δ F[1,20] = 7.71, Δ p = 0.012; VI: Δ F[1,20] = 7.10, Δ p = 0.015; VM: Δ F[1,20] = 5.36, Δ p = 0.031). After accounting for covariates, intramuscular fat of the VL, VI, and VM helped to explain 19.8%, 23.0%, and 5.9%

of additional variance in the lateral tibia, respectively. However, only the addition of intramuscular fat of the VL and VI were significant (VL: Δ F[1,20] = 5.37, Δ p = 0.031; VI: Δ F[1,20] = 6.55, Δ p = 0.019; VM: Δ F[1,20] = 1.36, Δ p = 0.258). In all significant cases for the medial patella, less intramuscular fat was associated with longer relaxation times (VL: ß =- 0.55, t = -2.78; VI: ß = -.53, t = -2.66; VM: ß = -.49, t = -2.32). This opposite effect was found in the lateral tibia where more intramuscular fat was associated with longer relaxation times (VL: ß =.48, t = 2.32; VI: ß = .52 , t = 2.56).

5.5.1.3 Between-limb Comparisons Effect Size Estimates for Strength, Intramuscular Fat, Volume and Fat-Cleared Muscle Volume

Metric	ACLR Group	Control Group
	Cohens *d*	Cohens *d*
Peak Torque	-0.49 [-1.09, 0.11]	0.34 [-0.25, 0.93]
Vasti Normalized Volume	-2.11 [-2.96, -1.26]	0.16 [-0.42, 0.74]
Vasti Fat-cleared Volume	-2.07 [-2.92, -1.23]	0.17 [-0.41, 0.76]
Vasti Intramuscular Fat	-0.06 [-0.64, 0.52]	-0.02 [-0.60, 0.56]

Table 18. Between-limb Comparisons in by Group Effect Size Estimates and 95% Confidence Interval's for Total Vasti Outcomes. Torque, volume, and fat units are in N·m/kg, mm³/kg, and %, respectively. Means, standard deviations, and test-statistics are located in Tables 11-13.

Metric	Muscle	ACLR Group Cohens *d*	Control Group Cohens *d*
Normalized Volume	VL	-1.43 [-2.15, -0.71]	0.17 [-0.41, 0.76]
	VI	-1.56 [-2.30, -0.82]	0.11 [-0.47, 0.69]
	VM	-1.92 [-2.73, -1.10]	0.05 [-0.53, 0.64]
Fat-cleared Volume	VL	-1.44 [-2.16, -0.72]	0.20 [-0.39, 0.78]
	VI	-1.50 [-2.23, -0.77]	0.11 [-0.48, 0.69]
	VM	-1.94 [-2.76, -1.12]	0.08 [-0.50 0.66]
Intramuscular Fat	VL	-0.22 [-0.80, 0.37]	0.04 [-0.55, 0.62]
	VI	0.01 [-0.57, 0.59]	0.01 [-0.57, 0.59]
	VM	0.16 [-0.43, 0.74]	-0.18 [-0.76, 0.41]

Table 19. Between-limb Comparisons in by Group Effect Size Estimates and 95% Confidence Interval's for Individual Vasti Outcomes. Torque, volume, and fat units are in N·m/kg, mm^3/kg, and %, respectively. Means, standard deviations, and test-statistics are located in Tables 11-13. VL indicates vastus lateralis; VI, vastus intermedius; VM, vastus medialis.

Muscle	Region	ACLR Group Cohens *d*	Control Group Cohens *d*
VL	Proximal	0.04 [-0.54, 0.62]	0.12 [-0.46, 0.70]
	Central	-0.30 [-0.89, 0.29]	0.07 [-0.51, 0.65]
	Distal	-0.15 [-0.73, 0.43]	-0.08 [-0.66, 0.50]
	Superficial	-0.23 [-0.82, 0.35]	0.06 [-0.52, 0.64]
	Deep	-0.20 [-0.78, 0.39]	0.02 [-0.56, 0.60]
VI	Proximal	0.23 [-0.35, 0.82]	0.18 [-0.40, 0.76]
	Central	-0.14 [-0.72, 0.44]	0.05 [-0.53, 0.63]
	Distal	0.11 [-0.47, 0.69]	-0.25 [-0.83, 0.34]
	Superficial	-0.07 [-0.65, 0.51]	-0.02 [-0.60, 0.56]
	Deep	0.15 [-0.44, 0.73]	0.06 [-0.52, 0.64]
VM	Proximal	-0.05 [-0.63, 0.54]	-0.19 [-0.78, 0.39]
	Central	0.05 [-0.54, 0.63]	0.07 [-0.51, 0.66]
	Distal	0.28 [-0.31, 0.87]	-0.38 [-0.97, 0.21]
	Superficial	0.12 [-0.46, 0.70]	-0.17 [-0.75, 0.42]
	Deep	0.20 [-0.39, 0.78]	-0.18 [-0.76, 0.40]

Table 20. Between-limb Comparisons in by Group Effect Size Estimates and 95% Confidence Interval's for Intramuscular Fat (%) by Region. Means, standard deviations, and test-statistics are located in Tables 14-15. VL indicates vastus lateralis; VI, vastus intermedius; VM, vastus medialis.

5.5.1.4 Between-limb Comparisons Effect Size Estimates for $T_{1\rho}$ and T_2

Location	Compartment	Region	ACLR Group Cohens d	Control Group Cohens d
Femur	Medial	Whole	0.63 [0.02, 1.24]	-0.24 [-0.83, 0.35]
		Anterior	-0.59 [-1.26, 0.08]	-0.03 [-0.71, 0.64]
		Combined WB	0.57 [-0.03, 1.18]	0.10 [-0.48, 0.69]
		Central-Anterior	-0.46 [-1.05, 0.14]	-0.14 [-0.72, 0.45]
		Central-Central	0.59 [-0.02, 1.20]	-0.16 [-0.75, 0.42]
		Central-Posterior	0.77 [0.14, 1.39]	0.30 [-0.29, 0.89]
		Posterior	0.32 [-0.27, 0.91]	-0.44 [-1.04, 0.15]
	Lateral	Whole	0.23 [-0.36, 0.82]	0.31 [-0.28, 0.90]
		Anterior	0.47 [-0.13, 1.06]	0.66 [0.05, 1.28]
		Combined WB	0.19 [-0.40, 0.77]	0.20 [-0.39, 0.78]
		Central-Anterior	-0.28 [-0.87, 0.31]	0.61 [-0.001, 1.22]
		Central-Central	0.01 [-0.57, 0.59]	0.12 [-0.46, 0.71]
		Central-Posterior	0.66 [0.04, 1.27]	0.22 [-0.37, 0.80]
		Posterior	-0.16 [-0.74, 0.42]	0.11 [-0.47, 0.69]
Tibia	Medial	Combined WB	0.31 [-0.28, 0.89]	0.03 [-0.55, 0.61]
		Central-Anterior	-0.78 [-1.41, -0.16]	-0.01 [-0.59, 0.57]
		Central-Central	0.24 [-0.35, 0.82]	-0.18 [-0.76, 0.41]
		Central-Posterior	0.56 [-0.04, 1.17]	0.22 [-0.36, 0.81]
	Lateral	Combined WB	0.28 [-0.31, 0.87]	0.19 [-0.40, 0.77]
		Central-Anterior	-0.45 [-1.04, 0.15]	0.02 [-0.56, 0.60]
		Central-Central	0.44 [-0.16, 1.04]	0.20 [-0.38, 0.79]
		Central-Posterior	0.32 [-0.27, 0.91]	0.24 [-0.35, 0.83]
Patella	Medial	Whole	0.96 [0.31, 1.61]	0.14 [-0.45, 0.72]
	Lateral	Whole	0.79 [0.17, 1.42]	0.12 [-0.46, 0.70]
Trochlea		Whole	0.69 [0.07, 1.31]	0.15 [-0.43, 0.73]

Table 21. $T_{1\rho}$ Relaxation Times Between-limb Comparisons in by Group Effect Size Estimates and 95% Confidence Interval's. Means, standard deviations, and test-statistics are located in Table 16. WB, indicates combined weight-bearing.

Location	Compartment	Region	ACLR Group Cohens *d*	Control Group Cohens *d*
Femur	Medial	Whole	0.82 [0.19, 1.45]	0.20 [-0.38, 0.79]
		Anterior	0.89 [0.19, 1.58]	-0.10 [-0.78, 0.57]
		Combined WB	-0.11 [-0.69, 0.48]	-0.04 [-0.62, 0.54]
		Central-Anterior	0.97 [0.32, 1.62]	0.30 [-0.29, 0.88]
		Central-Central	-0.11 [-0.69, 0.47]	-0.28 [-0.87, 0.31]
		Central-Posterior	-0.35 [-0.94, 0.24]	0.20 [-0.38, 0.79]
		Posterior	1.36 [0.66, 2.07]	0.27 [-0.32, 0.86]
	Lateral	Whole	0.89 [0.25, 1.53]	0.56 [-0.05, 1.16]
		Anterior	1.00 [0.35, 1.66]	0.48 [-0.12, 1.07]
		Combined WB	-0.37 [-0.96, 0.23]	0.61 [0.002, 1.22]
		Central-Anterior	-0.16 [-0.74, 0.43]	0.77 [0.14, 1.39]
		Central-Central	-0.42 [-1.01, 0.18]	0.50 [-0.10, 1.10]
		Central-Posterior	-0.30 [-0.89, 0.29]	0.11 [-0.48, 0.69]
		Posterior	0.81 [0.18, 1.44]	0.06 [-0.52, 0.64]
Tibia	Medial	Combined WB	-0.04 [-0.62, 0.54]	-0.28 [-0.87, 0.31]
		Central-Anterior	-0.21 [-0.80, 0.37]	-0.13 [-0.71, 0.46]
		Central-Central	-0.31 [-0.90, 0.28]	-0.23 [-0.82, 0.36]
		Central-Posterior	0.57 [-0.04, 1.17]	0.04 [-0.54, 0.62]
	Lateral	Combined WB	-0.34 [-0.93, 0.25]	0.53 [-0.07, 1.13]
		Central-Anterior	-0.51 [-1.11, 0.09]	-0.05 [-0.64, 0.53]
		Central-Central	-0.19 [-0.78, 0.39]	0.36 [-0.24, 0.95]
		Central-Posterior	0.76 [0.14, 1.38]	0.16 [-0.42, 0.74]
Patella	Medial	Whole	0.39 [-0.20, 0.98]	-0.09 [-0.68, 0.49]
	Lateral	Whole	-0.33 [-0.92, 0.26]	-0.06 [-0.64, 0.52]
Trochlea		Whole	0.28 [-0.30, 0.87]	-0.002 [-0.58, 0.58]

Table 22. T$_2$ Relaxation Times Between-limb Comparisons in by Group Effect Size Estimates and 95% Confidence Interval's. Means, standard deviations, and test-statistics are located in Table 17. WB, indicates combined weight-bearing.

6 Summary and Future Directions

6.1 Introduction

The goal of this dissertation was to identify origins and consequences of muscle dysfunction following ACL injury and ACLR. To do so, we utilized pre-clinical and clinical models, and implemented a variety of novel technological advancements to comprehensively assess intrinsic factors of muscle and joint health that haven't been fully characterized following ACL injury and ACLR. We also explored the relationship between these intrinsic factors of muscle, quadriceps strength, knee mechanics, and joint health including both subchondral bone architecture and cartilage degradation. Given the prevalence of early-onset PTOA following ACL injury and ACLR, understanding the extent of quadriceps dysfunction beyond strength and atrophy and its relationship with PTOA is crucial to understand disease mechanisms and identify targets for intervention. As such, the findings of this dissertation expand existing work in the fields of ACL injury and ACLR in the understanding of muscle and joint function. The following is a summary of each chapter that includes study outcomes, clinical implications, limitations, and future directions.

6.2 Summary

In **Chapter 3** we utilized a pre-clinical model to between understand the relationship between knee mechanics and subchondral bone architecture following ACL injury. Importantly, many pre-clinical models of ACL injury are considered transection models that mask the native

biological response to injury and confound observations. Here, we substantiated our model of non-invasive ACL injury that is clinically translatable and mimics the human mechanism of injury. As such, we ensure that our findings are truly a response to the ACL injury itself rather than any confounding variable that is introduced through surgical transection. The key findings of this study were that decreased knee flexion was related to subchondral bone plate porosity, an early surrogate measure of PTOA. Additionally, we observed significant variations in longitudinal joint kinematics and in both trabecular and subchondral bone. The implications and future directions of this study are vast. This is the first study to make a direct link between knee mechanics and an early indicator of PTOA. The clinical significance of this is that our model can be utilized to assess the efficacy of interventions aimed at improving or mitigating alterations in knee mechanics and subchondral bone architecture.

Chapter 4 was conducted in those with a chronic history of ACLR (2-5 years post-ACLR) and investigated *in vivo* vastus lateralis fascicle mechanics and knee joint mechanics during walking, and intramuscular fat fraction of the vastus lateralis. The goals of this study were to I) determine if abnormal fascicle mechanics were observed following ACLR II) understand the relationship between intramuscular fat and fascicle mechanics and III) understand the association between fascicle and knee mechanics. We found that following ACLR, patients exhibit between-limb strength and fascicle excursions (length and angle) that resemble those of healthy controls. However, we observed a smaller fascicle angle at peak KEM in the ACLR-involved limb. We also found the ACLR limb exhibited significant atrophy of the vastus lateralis but there were not any differences in intramuscular fat relative to the control limbs. All together, these data indicate that after ACLR, a muscle's overall change in shape (fascicle length and angle) is similar to healthy controls, however, there are fascicle angle deficits that occur when greatest demand is

placed on the quadriceps muscle that cannot be attributed to muscle atrophy or intramuscular fat. This data demonstrates at chronic time points following ACLR, there are alterations in knee mechanics consistent with an underloading pattern, significant muscle atrophy, and abnormal contractile behavior during walking at the time of peak demand on the quadriceps. Interestingly, this occurred without any observed strength deficits, suggesting that strength does not adequately portray quadriceps and knee function during walking. Clinically, this is impactful as it contributes to the growing body of literature showing that quadriceps weakness following ACLR is not isolated to strength deficits or muscle atrophy, and that there are intrinsic properties of muscle that contribute to global muscle dysfunction that warrant further investigation. In addition, this is the first study to measure intramuscular fat of the vastus lateralis using three-dimensional MRI following ACLR, a more sensitive measure of intramuscular fat than previous surrogate measurements of intramuscular fat using hyperechoic signaling captured via B-mode ultrasound.

Chapter 5 was conducted on the same patients as Chapter 4, which included those with a chronic history of ACLR (2-5 years post-ACLR). We investigated markers of muscle and joint health including muscle size, composition, and strength, cartilage proteoglycan density, collagen organization and water concentration. The goals of this study were to I) determine if quadriceps intramuscular fat was associated with cartilage composition II) determine if muscle size and intramuscular fat was associated with quadriceps strength III) to fully characterize the presence of intramuscular fat globally and regionally and IV) to fully characterize cartilage composition utilizing $T_{1\rho}/T_2$ quantitative imaging. We found that following ACLR, patients exhibit muscle atrophy of all vasti muscles but are able to achieve between-limb strength that resembles healthy controls. In addition, we did not find that intramuscular fat differed between-limbs or relative to

the Control group at this time point post-ACLR. We did observe alterations in various $T_{1\rho}/T_2$ ROI's in both the ACLR and Control groups. However, the most significantly notable changes with large effect sizes were localized in the ACLR-involved limb medial patella, aMF, pMF. Overall, the patients included in Chapters 4 and 5 were relatively healthy, participated in recreational activities and achieved adequate strength outcomes. Despite this, patients self-report dysfunction and exhibit muscle atrophy and degenerative changes in the cartilage matrix.

6.3   Limitations and Future Direction

6.3.1   Dissertation Limitations and Future Directions

Though the studies included in this dissertation contribute to the existing literature, there are several limitations to consider. Many of these limitations can be used to help guide future research aimed at understanding the complex interplay between factors effecting muscle and joint function, and precipitating PTOA development following ACLR.

Chapter 3 was conducted in a rodent model and characterized longitudinal joint kinematics and subchondral bone architecture following ACL injury. One of the primary limitations of the study was that we did not include metrics of muscle function or morphology and we did not directly assess cartilage structure or composition. Although joint kinematics were performed longitudinally, we did not have access to a scanner with capabilities to capture *in vivo* subchondral bone architecture. Pre-clinical models have unique advantages relative to clinical models, including the ability to probe multiple systems simultaneously and at multiple levels (i.e., macro- and micro-level). The data included in Chapter 3 were performed at the macro-level and included measurements of joint kinematics and subchondral bone architecture. However, probing systems at the micro-level such as biochemical and inflammatory signaling pathways

involved in muscle homeostasis, may help to understand the factors influencing both muscle and joint function after ACL injury. Additionally, this study was performed following ACL injury so how well our findings are exacerbated by surgical intervention (i.e., ACLR), warrants further investigation.

Chapters 4 and 5 were conducted in the same patients and explored fascicle mechanics, muscle strength, size, and intramuscular fat, and cartilage composition. Some of the primary limitations of these studies were that it was performed cross-sectionally, and we did not include measurements of neuromuscular function (e.g., electromyography, quadriceps activation). Future work would benefit from longitudinal study designs and the inclusion of neural pathways that have been shown to be disrupted post-ACLR. In addition, our mDixon MRI was primarily sensitive to intramuscular fat but there are other compositional components that can influence or interfere with contractile mechanics. Other imaging modalities such as mass spectrometry may be advantageous to fully characterize muscle composition and structure following ACLR. Specifically to Chapter 4, we did not capture fascicle mechanics throughout the range of a gait cycle and instead narrowed our assessment to an area of early stance where the quadriceps reached peak demand (peak KEM). Understanding if there are differences in fascicle mechanics throughout the gait cycle and over a range of activity types is important to understand the extent of fascicle abnormalities following ACLR. Additionally, muscle biopsies may advance our understanding of individual fiber contractile mechanics while permitting additional measurements of fiber properties such as fiber type and size. Specifically to Chapter 5, we did not include measurements of muscle function beyond muscle strength. However, understanding functional mechanics of the muscle and joint in relation to muscle and joint health is pivotal to understand the clinical meaningfulness of outcomes. While this study was one of the first to

investigate muscle size, intramuscular fat, and strength in relation to cartilage composition, future work should include assessments of both muscle and joint composition, structure, and function to understand the pathogenesis of PTOA more fully post-ACLR.

6.3.2 Technological Considerations and Future Directions

Although significant efforts were made to implement technologically advanced and novel two- and three-dimensional imaging modalities (video-based marker-less motion capture, B-mode ultrasound, mDixon MRI, and $T_{1\rho}/T_2$ MRI), each modality has inherent limitations that necessitate ongoing and dedicated research efforts.

Chapter 3s marker-less motion capture for instance encounters artifact and variability stemming from factors like subcutaneous fat, variations of movement in different planes of motion, and human-level labeling of images. To address these limitations, researchers have explored solutions such as dynamic x-ray radiography systems, multi-camera data collections to capture three-dimensional data, and increased personnel to improve labeling consistency. However, these options often rely on substantial resources (e.g., equipment cost, specialized lab personnel, implementation time). In order to ensure consistency in data measurements across studies, it is crucial to dedicate efforts towards developing low-cost alternatives for equipment and tools that can be employed across studies, animals, movement types, research sites, and personnel with a variety of skillsets. This approach would facilitate standardized methods and enhance the fields' ability to adapt to evolving technological advancements.

In **Chapter 4**, the utilization of *in-vivo* B-mode ultrasound imaging in the context of quadriceps fascicle mechanics represents a relatively new technological advancement in the field of rehabilitation sciences. Consequently, multiple limitations need to be considered. These

limitations encompass factors such as surrounding muscle tissue, probe placement, transducer orientation and compression, ultrasound settings, and movement artifacts. Additionally, limited research exists on the translation of fascicle measurements to the muscle-fiber level. If ultrasound usage for capturing fascicle mechanics becomes more widespread, it will be imperative to develop methods that standardize these factors to ensure consistent and accurate measurements, as well as establishing an understanding of the relationship between fascicle and muscle-fiber mechanics.

The application of mDixon MRI in **Chapters 4 and 5** involves a technique that leverages unique chemical properties to capture the relative amount of fat in regions of muscle. However, measurements obtained through this modality are susceptible to artifacts by surrounding tissues, specific scanner parameters and protocol, patient positioning, and data processing methods. Lastly, **Chapter 5** is the first experimental study with subjects at the University of Michigan to utilize $T_{1\rho}/T_2$ imaging to measure properties of the knee cartilage matrix. $T_{1\rho}/T_2$ quantitative imaging, similar to mDixon MRI, is heavily influenced by imaging protocols, and data processing methods. The field as a whole would greatly benefit from developing standardized methods for processing and analyses across all imaging modalities. While some variation and user-preference may be expected, establishing a degree of standardization grounded in scientific rigor will enable the field to evolve alongside technological advancements, particularly in computer and data science, and engineering. This standardization will improve consistency in interpretation across studies, ultimately expediting scientific research and development aimed at improving patient care.

Appendices

Appendix A: Institutional Animal Care and Use Committee Forms (Chapter 3)

UCONN
UNIVERSITY OF CONNECTICUT

R. Holly Fitch, Ph.D.
IACUC Chair
860-486-2554
roslyn.h.fitch@uconn.edu

DATE: January 8, 2018

TO: Lindsey Lepley, Ph.D.
           Department of Kinesiology, Unit 1110

FROM: R. Holly Fitch, Ph.D.

SUBJECT: Notice of IACUC Approval for Protocol No. A17-042

This letter serves as written notice of animal use **APPROVAL** by the IACUC on January 8, 2018.
Please note that at the October 30, 2017 meeting, the IACUC determined that the protocol required
modifications to secure approval that now have been addressed. Please refer to the assigned
protocol number for all animal orders and future correspondence with the IACUC; cage cards
should contain this protocol number. Please advise us in the future of any necessary corrections or
revisions by completing the appropriate form at http://research.uconn.edu/iacuc/iacuc-forms/.

Please Note: All investigators are required to make study records available for inspection during
normal business hours. If study records are kept in locked facilities, a member of the research staff
must be designated as the official contact person for record inspection.

If you have added new personnel (students, post-docs, or faculty members) to the protocol,
you must contact ACS Veterinary Staff (acstraining@uconn.edu) to schedule the
appropriate training in Basic Animal Handling and if assisting or conducting surgery,
training in Anesthesia and Aseptic Technique, etc.

This institution has an Assurance of Compliance on file with the Office of Laboratory Animal
Welfare, National Institutes of Health (Assurance #A3124-01, 2/28/2020).

Funding Source:
> 1. NIH: "Influence of Eccentric Exercise on Muscle and Joint Health Following ACL
>   Injury" (pending)

All animal use protocols must be reviewed annually from the date of IACUC approval; you will
receive an e-mail notice requesting an annual update. Thank you for your efforts to help keep the
University in compliance with all animal welfare regulations.

Office of the Vice President for Research
Research Compliance Services
438 WHITNEY ROAD EXTENSION, UNIT 1246
STORRS, CT 06269-1246
PHONE 860.486.8802
FAX 860.486.1044
compliance.uconn.edu
An Equal Opportunity Employer

Title: "Influence of Eccentric Exercise on Muscle and Joint Health
                Following ACL Injury"
Species: Rat
Date Approved: January 8, 2018 – January 7, 2021
Protocol #:   A17-042

c: Craig Denegar, Department Head, Dept. of Kinesiology
        IACUC Designated Reviewer
        Ramaswamy Chidambaram, Animal Care Services
        Sheryl Lohman, Animal Care Services
        William Field, Environmental Health and Safety
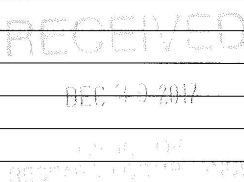
University of Connecticut

IACUC-1 Protocol Application for Live Animal Care and Use
Institutional Animal Care and Use Committee, Office of Research Compliance
Whetten Graduate Center, Rm #214, 438 Whitney Road Ext., Unit 1246 Storrs, CT 06269-1246 860-486-8802

Sample language and additional guidelines for completing protocol forms are listed on the <u>Protocol Instructions</u> page.

Section I: PI (Principal Investigator) and Laboratory Information

Principal Investigator (PI) Lindsey K Lepley PhD ATC
Department: Kinesiology
E-mail: lindsey.lepley@uconn.edu
Unit #: 1110
Phone #: 860-486-5322
Emergency contact information (please provide cell phone#): 989-859-2950

Section II: General Protocol Information

Project Title: Influence of Eccentric Exercise on Muscle and Joint Health Following ACL Injury
Submission type: ☒New ☐ Three year Renewal or ☐Modification of approved submission #
Type of project: ☒ Research ☐Teaching (course #:) ☐Public Service ☐Field research Check All That Apply
Anticipated project start date: 04/01/2018
(If different than above) When will animal work on this project begin?
Species: Rattus, Long Evans
Total animal number: 152

Section III: Funding and Collaboration

A. **Project Funding Status**
Funding sources may include internal (department, internal grants) or external (NIH, USDA, NSF etc.)

Check all that apply:

	Status	Name of granting agency or other source (department, institution, etc.)	Title of award (s) *or* FRS# (if internally funded)
	Fully or partly funded by federal grant		
X	Pending funding by federal grant	NIH, K01(AR071503-01)	Influence of Eccentric Exercise on Muscle and Joint Health Following ACL Injury
	Funding to be provided by University of CT		
	Funding provided by other organization		
	Other: describe how animal expenses will be covered:		

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B. Collaboration:
   Will any live animal work for this project be performed at a facility or institution other than the University of Connecticut Storrs campus (excluding the receipt of animals or tissues from approved vendors)?

   ☒ No   ☐ Yes   If Yes, complete the following:

   1. Which institution/facility will have ownership of the animals?
      ▶
   2. What is the nature of the collaborative work being performed, including where and with whom work will be performed?
      ▶
   3. If the collaborative work is being performed as part of an approved IACUC protocol at another institution, please include a copy of the approval letter with this protocol submission.
      ▶

Section IV: **Project Overview**

A. **Summarize your project. It may be helpful to think about how you would describe your project to the Media, or to someone with a high school education. Technical language from grant submissions is not acceptable in this section. Be sure to include how live animals will be used to contribute to your teaching or research goals and how this project will advance scientific knowledge or otherwise benefit human or animal health or well-being.**

   ▶The central tenant of this K01 is to identify rehabilitation strategies capable of promoting muscle and joint health after anterior cruciate ligament (ACL) injury. To date, concentric exercise (muscle strengthening during the shortening of contraction) is the standard mode of exercise prescription after ACL injury. Yet, multiple systematic reviews have shown that concentric exercise does not restore muscle strength and does not deter the onset of post-traumatic osteoarthritis (PTOA). Emerging evidence suggests that mechanical stress achieved via eccentric exercise (muscle strengthening during the lengthening mode of contraction) is required to initiate strain-sensing molecules that promote the recovery of muscle. We have recently demonstrated that embedding eccentric exercise into a rehabilitation program early after ACL reconstruction promotes the recovery of quadriceps strength better than concentric exercise. Though promising, eccentric exercise is often avoided clinically, as the notion that eccentric exercise is associated with muscle injury has been perpetuated in the literature, and the underlying benefits of eccentrics to muscle remain undefined. Further, the ability of eccentric exercise to prevent the development of PTOA in a high-risk population has never been studied. To provide the evidence-based data needed to support the incorporation of eccentric exercise into rehabilitation, we will establish a non-invasive, clinically-translatable, ACL injury in a rat model and describe the time course of biomechanical alterations, inflammatory response and PTOA progression (aim 1). We will then use this model to report the effectiveness of eccentric exercise to treat muscle weakness (aim 2) and promote bone and cartilage health after ACL injury (aim 3). The central hypothesis is that eccentric exercise will attenuate deficits in neural activity and alterations in muscle morphology, thereby restoring quadriceps strength and promoting joint health more effectively than the current standard of care of concentric exercise after ACL injury. The rationale for conducting this *in vivo* research, which establishes the physiological benefits of this rehabilitation strategy, is to ultimately inform the development of future clinical trials.

B. Is your project an extension of previous work? If this project is an extension of your previous work or the work of others, briefly explain why additional work is being done.

▷ To promote the recovery of muscle, emerging evidence suggests that mechanical stress achieved via eccentric exercise is required to promote tissue growth. Given the unique capability of eccentric exercise to mechanically engage muscle, the immediate incorporation of eccentric exercise into rehabilitation is likely an effective intervention to promote the recovery of muscle strength. In fact, recent evidence suggests that eccentric exercise can be safely employed after ACL reconstruction, and that eccentrics is particularly beneficial to treating specific deficits that occur after ACL injury (Figures 1A, 1B). My doctoral work directly supports this premise, as this study was the first to directly compare eccentric to concentric exercise after ACL reconstruction and demonstrate that eccentric exercise is beneficial to muscle strength and neural activity (Figure 1A). Others have also found that eccentric exercise positively influences muscle volume in patients post-ACL reconstruction compared to patients receiving concentric exercise (Figure 1B). While this work provides important preliminary data, to counteract the clinical belief that eccentric exercise is harmful, further efforts are needed to solidify the concept that eccentric exercise will promote the recovery of muscle after ACL injury. Hence this proposed research will contribute significantly to scientific knowledge by using an animal model of ACL injury to directly compare the underlying physiological benefits of eccentric exercise to concentric exercise after ACL injury.

Figure 1A: Comparison of Quadriceps Strength (Nm)

Figure 1B: Comparison of Quadriceps Hypertrophy (% change)

■ eccentric   ▨ concentric   * P<0.05

Section V: Request for Animal Use

A. Species Information:

1. Privately owned animals. If you are using any privately owned animals for research, teaching, or demonstration purposes, you must *submit a completed copy of Appendix B* with this protocol submission.

2. Species, location and source. To list additional species or strains, add lines to Table
Occasionally, it is not feasible to list all species and/ or strains that may be associated with a project (such as in field studies where other species may be unintentionally captured, or if a large number of transgenic mouse models will be used). Please provide a brief explanation if all species/strains cannot be listed:

Species/Strain	Source(s) of animals	# required to meet research/teaching objectives
Rattus, Long Evans	ACS approved vendor	152

3. Age and sex of animals on study: Indicate the sex and approximate age(s) of animals to be used in your research, if possible. Also identify in your description use of any breeder animals, neonates, feti, and all culled animals:

▷ For Aim 1, we plan to use 56 Long Evans rats age 16 wks at time of injury (28 males, 28 females). For aims 2 & 3 we plan to use 96 Long Evans rats age 16 wks at time of injury (48 males, 48 females).

4. Animals with special requirements: Do any of the animals have a phenotype that may cause a known painful or distressful physical, behavioral, or physiological condition (e.g. susceptibility to a particular illness, weakened immune system) or that may require special precautions to be taken by caretaker staff for the animal's benefit (e.g. requiring vaccination of staff, special hygiene precautions)?
☒No ☐Yes: If "Yes", describe condition(s) and any special caretaker requirements
▷

5. Genetically modified animals: Will this project use any transgenic, knock-out, knock-in, floxed, cloned, or otherwise genetically modified stock or strain of animal?
☒No   ☐Yes: If "Yes," complete the following:
a. Identify which ones:
▷
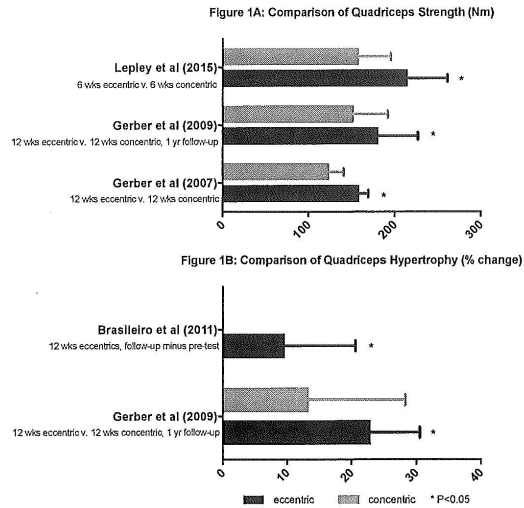
b. Do any of the genetic modifications make the animals susceptible to a known disease condition, or otherwise contribute to conditions of pain or distress?
☐No ☐Yes: If "Yes", describe:
▷

c. List all known phenotypes of this strain (e.g. prone to seizure, splayed legs, etc.)
▷

d. Ensure you have completed all necessary documentation for use of genetically modified animals as required by the Institutional Biosafety Committee (IBC) www.ibc.uconn.edu .

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Section VI: Justification of Animal Use and Numbers

A. Justification of Animal Use:

1. Rationale for using live vertebrate animals in this project:
   What is the reason that live vertebrate animals are necessary for this project? (Complete Table)

   Check all that apply:

x	The complexity of the processes being studied cannot be replicated, duplicated, or modeled in simpler living systems, such as in plants, insects, or other invertebrates.
x	There is not enough information about the process being studied to design in-vitro or non-living models
x	Existing in-vitro or non-living processes cannot produce the required results (e.g., cell culture for monoclonal antibody production, computer modeling of protein synthesis, etc)
	Preclinical studies in living vertebrate animals are necessary prior to human testing
	This is a behavioral, learning, or development study: a whole living system is required
	This is an ecological or field study
	The animals will be used for teaching/ demonstration purposes
	Other- please describe:

2. Appropriateness of species / strain selected:
   For each species and strain listed in Section V.A.2, please describe the rationale in the table below:

Enter species name across top, then check all rationale that apply for that species	Species/ strain 1:Rattus, Long Evans
This is a new model with untested properties	No
A large database exists for this species/ strain which will allow comparisons to previous data	Our work will use male and female Long Evans rats at 16 weeks of age, as these rats are skeletally mature, and this strain of rat has been used extensively for research involving exercise, muscle and joint injury and adaptation.
The anatomy, genetics, physiology, phenotype, or behavior of the species is uniquely suited to the proposed study	Due to the difficulties associated with controlling the physiological effects of exercise in humans, and the need to directly determine the effect of an ACL injury and exercise on alterations in neural activity and muscle morphology and joint health, this project will utilize Long Evans rats. There currently is no alternative computer, invertebrate, or in vitro models available that will allow us to perform the proposed experiments. The number of rats required to perform the proposed experiments is kept to a minimum by calculating the lowest number needed to achieve statistical power.
This is the phylogenically least complex model that will provide adequate tissue, size, or anatomy for the proposed study	Yes, Long Evans rats are excellent for exercise protocols and are large enough to instrument. Mice are too small for our instrumentation.
The results will be directly applicable to the health or care of this species	PTOA is a major cause of lifetime disability, for which no cure currently exists. PTOA is especially problematic following ACL injury, where regardless of treatment, PTOA affects over 50% of ACL injured limbs. Using a rodent model that closely resembles human ACL injury, the proposed studies will elucidate whether eccentric exercise is more effective than concentric exercise at promoting muscle and joint health after ACL injury for the purpose of providing clinicians with evidence-based treatment options for the nearly 300,000 Americans who experience ACL injury each year.
Other: please describe additional rationale used to select the species and strain requested	N/A

B. Justification of Animal Numbers:
   1. What was the method(s) used to determine how many live animals are required for this study? Check all that apply, and supply additional information where asked to do so:

	This is a field study (e.g. a mark/recapture study for estimating population size/trends	

	or survival) in which the nature of the research requires as many animals as can be located)	
	Numbers were mandated by FDA or other government agency (e.g. GLP work)	Which agency?
x	**Numbers were based on results of a pilot study**	**Please reference study:** • Lepley, L.K., McKeon, P.O., Fittzpatrick, S.G., Beckemyer, C.L., Uhl, T.L., Butterfield, T.A., 2016. Neuromuscular Alterations After Ankle Sprains: An Animal Model to Establish Causal Links After Injury. Journal of Athletic Training 51, 797-805.
x	**Numbers were based on previous research or experiment by self or others**	**Please reference experiment:** • Bigoni, M., Turati, M., Gandolla, M., Sacerdote, P., Piatti, M., Castelnuovo, A., Franchi, S., Gorla, M., Munegato, D., Gaddi, D., 2016. Effects of ACL reconstructive surgery on temporal variations of cytokine levels in synovial fluid. Mediators of Inflammation 2016, 8243601. • Haslauer, C.M., Proffen, B.L., Johnson, V.M., Hill, A., Murray, M.M., 2014. Gene expression of catabolic inflammatory cytokines peak before anabolic inflammatory cytokines after ACL injury in a preclinical model. Journal of Inflammation 11, 34. • Jones, M.D., Tran, C.W., Li, G., Maksymowych, W.P., Zernicke, R.F., Doschak, M.R., 2010. In vivo microfocal computed tomography and micro–magnetic resonance imaging evaluation of antiresorptive and antiinflammatory drugs as preventive treatments of osteoarthritis in the rat. Arthritis & Rheumatism 62, 2726-2735. • Lepley, L.K., McKeon, P.O., Fittzpatrick, S.G., Beckemyer, C.L., Uhl, T.L., Butterfield, T.A., 2016. Neuromuscular Alterations After Ankle Sprains: An Animal Model to Establish Causal Links After Injury. Journal of Athletic Training 51, 797-805. • Lepley, L.K., Wojtys, E.M., Palmieri-Smith, R.M., 2015. Combination of Eccentric Exercise and Neuromuscular Electrical Stimulation to Improve Quadriceps Function Post-ACL Reconstruction. The Knee 22, 270-277. • Mendias, C.L., Lynch, E.B., Davis, M.E., Enselman, E.R.S., Harning, J.A., DeWolf, P.D., Makki, T.A., Bedi, A., 2013. Changes in Circulating Biomarkers of Muscle Atrophy, Inflammation, and Cartilage Turnover in Patients Undergoing Anterior Cruciate Ligament Reconstruction and Rehabilitation. The American journal of sports medicine 41, 1819-1826. • Wurtzel, C.N., Gumucio, J.P., Grekin, J.A., Khouri, R.K., Russell, A.J., Bedi, A., Mendias, C.L., 2017. Pharmacological inhibition of myostatin protects against skeletal muscle atrophy and weakness after anterior cruciate ligament tear. Journal of Orthopaedic Research Epub ahead of print.
x	**Numbers were calculated using a statistical formula**	**Please reference the name of the formula(S):** Based on previous animal (Bigoni et al., 2016; Haslauer et al., 2014; Jones et al., 2010; Lepley et al., 2016; Wurtzel et al., 2017) and human studies (Lepley et al., 2015; Mendias et al., 2013), effect sizes were calculated for each of the outcomes, and regardless the type of study and outcome, they all can be classified as large effect sizes ($d \geq 0.8$,(Cohen, 1988), alpha=0.05). **Aim 1:** The goal of this aim is to test the null hypothesis that the means over time are equal for the different outcomes. The computations assume a small intraclass correlation of 0.15. (Mendias et al., 2013) For the 6 time points, 56 rats will be needed (48 ACL injured rats, 8 controls [requesting additional ~20% for attrition]). **Aim 2 & 3:** The goals of these aims are to test the null hypothesis that the 4 rat group means are equal for different outcomes. With a proposed

		sample size of 24 rats for each group (12 males, 12 females [requesting additional ~20% for attrition]), the study will have power of 80% to yield a significant result. This computation assumes that the standardized mean difference is 0.8 and the common within-group SD is 0.5.(Lepley et al., 2016; Lepley et al., 2015; Wurtzel et al., 2017).
x	**Numbers were calculated via consultation with a statistician**	**Who was consulted, and on which date(s):** Dr. Tania Huedo-Medina, Associate Professor of Allied Health Sciences (continuous consultation spring/summer 2017 during development of proposed research).
	Numbers are based on expected student enrollment: reflects animal/student ratio required for effective teaching	
	This is a breeding or holding protocol, and numbers represent the estimates of offspring that will be produced and/or animals that will otherwise need to be held while not on study	
	This is a pilot project which will be used to refine future experiments	
	None of the above methods could be used to determine numbers, and the numbers requested represent the best estimates in the PI's professional judgment	**Please explain why none of the above methods could be used, and how the final numbers were determined:**

Please describe how you calculated the animal numbers, either through a narrative or using a simple table that tabulates across studies and/or groups. IF THIS IS A FIELD STUDY IN WHICH NUMBERS ARE OPEN ENDED, SKIP THIS STEP. Make SURE the numbers and terminology for individual studies/groups is consistent with the description you provide in VIII.C, and confirm that numbers match totals provided in Section II and V. Also confirm numeric consistency with percentages in the Pain and Distress table below (VII), including ALL animals used (e.g., culled pups and spent breeders, typically category B). Again, culls and breeders MUST be reflected in Total Animal Number, here and elsewhere. (Suggest use Microsoft Word Insert Table feature or similar quick tool.)

Aim 1: We plan to use 56 Long Evans rats age 16 wks at time of injury (28 males, 28 females [power detailed above in justification of numbers]). 48 rats will undergo the non-invasive ACL injury (right hindlimbs only), followed by normal cage activity and data collections at 1, 3, 7, 14, 28 or 56 days after non-invasive ACL injury (n=8 rats/time point [4 males, 4 females]). 8 (4 males, 4 females) rats will be uninjured and euthanized at 24 wks of age.

Aims 2 & 3: We plan to use 96 Long Evans rats age 16 wks at time of injury (48 males, 48 females [power detailed above in justification of numbers]). Rats will be randomly assigned (24 per group [12 males, 12 females]) to an eccentric (downhill walking), concentric (uphill walking), injury model (no exercise), or control group (no injury, no exercise) and the proposed interventions.

<u>Section VII:</u>   Pain and Distress Classification* by Species

A. <u>Categories:</u>

Insert the common names of each species used and enter the ~% of Animals for each pain classification Category. Count each animal only once. For example, if you are working with rabbits, and some will undergo procedures classified as Category B at some point during the experiment, but then the *same animals* will undergo Category D classification procedures at a later time point, classify all of these animals as Category D. (If more than 4 species will be used, copy and paste Table and note that added as an Appendix.)

A detailed guide for using the USDA's Pain Classification system can be found at:
http://oacu.od.nih.gov/ARAC/documents/USDA_Reports.pdf

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Common name of species used: (Place animals in "highest" USDA category.)	Species 1: Rattus, Long Evans
% Category B: Animals being bred, conditioned, or held for use in teaching, testing, experiments, research or surgery, but not yet used for such purposes.	n/a
% Category C: Animals upon which teaching, research, experiments, or tests will be conducted involving either no pain/distress, or only momentary/transient pain/distress.	n/a
% Category D: Animals used for teaching, research, experiments, testing or surgery that will involve accompanying pain or distress and for which appropriate . anesthetic, analgesic, or tranquilizing drugs will be used.	21%
% Category E: Animals used for teaching, research, experiments, testing or surgery that will involve accompanying pain or distress for which the use of appropriate anesthetic, analgesic, or tranquilizing drugs will adversely affect the procedures, results, or interpretation of the teaching, research, experiments, testing or surgery.	79%

1. Category D or E:
    If any of the above procedures fall into Category D or E, then you must *complete Appendix E*

2. Category E:
    If any of your classifications above include Category E procedures, you must *scientifically justify* below, the withholding of analgesia, anesthesia, tranquilizers, medical treatments, or other methods to alleviate pain/distress; include scientific references:
    ▶ Analgesics will be withheld as pain medications have been known to influence neural activity and the inflammatory process, two primary outcomes measures of this proposed work. Further, humans after ACL injuries may or may not receive analgesics (i.e. taking analgesics is not the standard of care). Hence withholding analgesics is in-line with the current standard of clinical practice.

    In-line with our previous work, analgesics will be provided after EMG instrumentation (Lepley, L.K., McKeon, P.O., Fittzpatrick, S.G., Beckemyer, C.L., Uhl, T.L., Butterfield, T.A., 2016. Neuromuscular Alterations After Ankle Sprains: An Animal Model to Establish Causal Links After Injury. Journal of Athletic Training 51, 797-805). To ensure that analgesics will not confound our measures, rats will be given 4 days of rest post-EMG implantation prior to the collection of any neural data for aim 2.

B. Overall Consideration of Sources of Stress and Distress:

   According to the principles of Refinement, the IACUC must identify all potential sources of stress and distress. *(These may include but are not limited to, stressors such as: food or fluid restriction, painful or distressful blood or tissue collection, noxious stimuli, painful surgery, separation of pre-weans or fledged animals from their mother, environmental distress, forced exercise, disease conditions, deliberate exposure to predator scents, exposure of non-acclimated animals to human handling, excessive noise, etc.)*

   Will animals in the proposed study be exposed to any factor(s) that may induce physiological or behavioral stress or distress above and beyond that which is associated with normal pet ownership or basic husbandry procedures or natural behaviors defined for the species?
   ☐No   ☒Yes: if "Yes," *complete table below:*

   Stressful and Distressful Procedures

Check all that apply	Procedures	If you checked this item, you must complete and submit the appropriate Appendix form or include additional information as identified below.
☐	Restrainers: metabolism cages, vests, harnesses, or slings, etc.	*Describe:*▶
☒	Potential stressors: food or water restriction, noxious stimuli, electrical shock, environmental stress, forced exercise, etc.	*Describe:*▶ *The use of electrical shock during treadmill walking is a potential stressor. An ACL injury is created in rats and could cause stress/distress.*
☐	Invasive methods of animal identification (tattoos, brands, implants, ear tags, ear notches or punches, toe clipping, tail biopsy genotyping etc.)	*Describe:*▶
☒	Injections or Inoculations (drugs, substances, vaccines etc.)	*Compounds and procedure(s) used must be described in Section VIII.A.1 below*
☒	Blood Collection	*Procedure(s) used must be described in Section VIII.A.4*
☐	Terminal Tissue Harvest: e.g. perfusion, exsanguination under anesthesia	*Ensure procedure is referred to by name in Section VIII.C below and is included as the method of euthanasia in Section X.C.*
☒	Non-Survival Surgery: i.e. surgical procedures in which an animal will be maintained in a plane of anesthesia for more than a few minutes from which it will not recover (e.g. non survival neuronal recordings, teaching surgical technique)	*Describe in APPENDIX G Ensure procedure is referred to by name in Section VIII.C below*
☐	Survival Surgery (Minor)	*Describe in APPENDIX G Ensure procedure is referred to by name in Section VIII.C below*
☒	Survival Surgery (Major)	*Describe in APPENDIX G Ensure procedure is referred to by name in Section VIII.C below*
☐	Exposure to Radiation or Lasers	*APPENDIX D Describe:*▶
☐	Exposure to Toxins (biological or chemical)	*APPENDIX D Describe:*▶
☐	Injections with or exposure to human cells (e.g. ESC)	*APPENDIX D Describe:*▶
☐	Injections with or exposure to biological materials of animal origin (e.g. murine cells)	Describe:▶
☐	Injections with or exposure to Infectious Agents (e.g. human or animal pathogens, viral vectors, etc.)	APPENDIX D

		Describe: ▶
☐	Transgenic or Knockout Animals	Ensure that animals are described in Section V.A.5

Contact the IBC to complete the necessary documentation |

C. Recognition and Assessment of Stress and Distress: For all procedures, including those listed above: Please identify any <u>expected</u> potential indicators of stress and distress for this project, and explain how the indicated conditions will be monitored. Examples include, but are not limited to: reduced activity, weight loss, abnormal posture, etc.
         ▶ All studies will be conducted in a manner to minimize discomfort, distress, pain, and injury while ensuring the scientific integrity of the studies. Specifically, throughout the protocol, each rat will be closely monitored for their level of responsiveness, grooming, and feed and water consumption. If a rat fails to meet these criteria, taken as an indication of distress, a veterinary consult will be conducted. Weights will also be continuously monitored throughout the protocol. During treadmill walking, the PI will closely monitor the rats for humane endpoints and stoppage of walking. Unwillingness to exercise will be monitored by the PI, and exercise will be discontinued if the rat is unable to keep pace with the treadmill, or if repeated electrical stimuli (e.g. more than 4 times per minute) is needed to elicit compliance with exercise intensity.

D. Refinement: For all procedures, including those listed above: Describe how you have incorporated the principle of refinement in order to reduce pain and distress. Examples include, but are not limited to: use of analgesia, acclimation and training of animals, catheterization for frequent blood collection, etc.
  ▶ We subscribe to the principle of refinement, and it is our goal to minimize any potential pain and distress during our experiment. There are currently no alternative computer, invertebrate, or in vitro models available that will allow us to perform the proposed experiments. The number of rats required to perform the proposed experiments is kept to a minimum by calculating the lowest number needed to achieve statistical power.

Analgesics will be withheld after ACL injury as pain medications have been known to influence neural activity and the inflammatory process, two primary outcomes measures of this proposed work. Further, humans after ACL injuries may or may not receive analgesics (i.e. taking analgesics is not the standard of care). Hence witholding analgesics is in-line with the current standard of clincial practice. To minimize distress, the use of moistened food, hydrogel cups, and palatable food treats may also be used after ACL injury and EMG electrode placement.

It is possible that a rat may experience an accidental injury (e.g. foot injury) during treadmill walking. In this scenario, exercise will be stopped if the rat is unable to continue exercising without an antalgic gait (i.e. antalgic gait due to foot injury, not due to ACL injury where hindlimb gait alterations are expected). Exercise will also be stopped due to poor performance (e.g. unwillingness to exercise). Unwillingness to exercise will be monitored by the PI, and exercise will be discontinued if the rat is unable to keep pace with the treadmill, or if repeated electrical stimuli (e.g. more than 4 times per minute) is needed to elicit compliance with exercise intensity.

<u>Section VIII</u>: Specific Procedures and Studies

A. <u>Invasive procedures:</u>

    1. Substance Administration: including anesthetics or analgesics used for surgery, will any drug or research material be given to the animals?

        ☐ No, If no, SKIP TO QUESTION 4.
        ☒Yes: List below including names, dosages, route, and site of delivery of the vehicle, drug or agent
        ▶ Anesthesia will be induced using an induction chamber with 5% isoflurane and 1L/min oxygen, and maintained via a nose cone with 1-3% isoflurane and 500mL.

        Rats will also be given Buprenorphine injection (0.02 mg/kg, subcutaneous injection) after EMG implantation with additional Buprenorphine injections every 8-14 hours thereafter for 72 hours.

        Alternatively, Meloxicam may also be used for 72 hours [3 total doses [dose 1 is administered pre-operatively], 1-2 mg/kg subcutaneous injection, ACS SOP #2003].

    2. Non-Pharmaceutical Grade Compounds: *The 2011 Guide (pg. 31)* states, "[*Pharmaceutical grade*

chemical compounds] should be used, when available, for all animal related procedures". USDA APHIS and OLAW define pharmaceutical grade compounds as "a drug or compound that can be purchased from a medical or veterinary drug supplier in a formulation that is ready-to-use in living vertebrate animal subjects; including those intended for use as investigational agents for clinical purposes and in terminal studies". "USP" is not equivalent to "pharmaceutical grade."

a. Does this study involve injections of non-pharmaceutical grade chemicals /substances?

☒No All chemical compounds used will be human or veterinary pharmaceutical grade.

☐ Yes Non-pharmaceutical grade chemicals or substances will be used because:

(Check all that apply)

	Pharmaceutical grade compound is not available from a veterinary or medical supplier
	Pharmaceutical grade compound is not available from a veterinary or medical supplier in the needed concentration or formulation
	The compound is required in order to produce data that is comparable to previous year's data
	Reagent grade compound is more pure than pharmaceutical grade compound
	Non-pharmaceutical grade compounds are necessary to meet the scientific goals of the study. Briefly explain: ▶

b. Please provide a written SOP (either below, or attach as an appendix) describing how the compound is prepared and stored. Be sure to include the following in your description:

- Chemical compound concentration (in units or %)
- Vehicle used
- How sterility is achieved (e.g. filtered, autoclaved)
- How labeled to include: Date compounded, by whom, shelf life and expiration date
- Storage requirements
- Assessment of pH (pH test paper is appropriate)

▶

3. Controlled Substances: Does the proposed work include use of any federal or state controlled substances?

☐No
☐Yes, under the handling and direction of a veterinary professional only
☒ Yes and the <u>PI or named protocol personnel</u> are licensed and store and/or administer the substances

DEA/CT license numbers of licensee:▶ Dr. Lepley has a DEA license (CSL#0001125)
Date issued:▶   10/06/16

The state of CT now requires you to provide a list of research personnel who are authorized by you to be named as handlers of the drugs under your license. You may email them this list at: <u>drug.control@ct.gov</u>

4. Blood collection: Does the proposed study involve survival collection of blood from a live animal?

☐No   ☒Yes: Describe procedures below, including route, site of collection, any use of catheterization, frequency, volume, and animal recovery methods that will be used.

▶ For aim 1, serial serum measures of inflammatory biomarkers will be collected via the dorsal pedal vein or the tail vein (e.g. tail vein prick method). Rats will be restrained and the dorsal pedal vein or the tail vein will be located. The area will then be cleansed with alcohol and the vein will be punctured with a 23G needle if the dorsal pedal vein method is used or a sterile scalpel for the tail prick method. Blood will be collected via a capillary tube (approximately 2ml volume) and slight pressure will be applied to the area to stop the bleeding.

To continuously monitor the inflammatory response in rats at at 1, 3, 7, 14, 28, or 56 days after ACL injury, and ensure that the inflammatory response in animals euthanized at early time points is similar to those euthanized at later dates, live animal data collections are required.

In animals that are alive for 56 days, blood will be collected n=6 times (1, 3, 7, 14, 28, or 56 days after ACL injury). In animals that are alive for 28 days, blood will be collected n=5 times (1, 3, 7, 14, 28 days after ACL injury). In animals that are alive for 14 days, blood will be collected n=4 times (1, 3, 7, 14 days after ACL injury). In animals that are alive for 7 days, blood will be collected n=3 times (1, 3, 7 days after ACL injury). Lastly in animals that are alive for 3 days, blood will be collected n=2 times (1 and 3 days after ACL injury) and animals that are alive for 1 day will undergo a single data blood collection.

5. Tissue collection: Does the proposed study involve survival collection of tissue(s) from a live animal?

☒No
☐Yes, for genetic biopsy such as ear punch or tail snip with anesthesia – *Describe in Appendix G* if different from NIH defined practices for rodent genetic biopsy (link referenced below)
http://oacu.od.nih.gov/ARAC/documents/Rodent_Genotyping.pdf

☐Yes, for other survival collection of tissue under anesthesia Ensure procedures are *listed by name* in Section VIII.C and *complete Appendix G* .

6. Surgical procedures: Does the proposed study involve any incisions, or abrading, or other surgical procedures on live vertebrate animals?

☐ No

☒Yes, for survival procedures (animal is expected to recover consciousness following the procedure) Ensure that procedures are *listed by name* in Section VIII.C, and *complete Appendix G.*

☒Yes, for non-survival surgeries where animals will be maintained in a plane of anesthesia for more than a few minutes (e.g. non-recovery neuronal recordings): Ensure procedures are *listed by name* in Section VIII.C and *complete Appendix G: Sections I,II & III only).*

☐Yes, for terminal tissue harvest procedures (e.g. perfusion, terminal tissue harvest, cardiac puncture): Ensure procedures are *described in* Section VIII.C. *DO NOT complete Appendix G*

B. **Specific types of studies:**

1. Does the proposed study involve the experimental modification and/or observation of animal behavior?
   ☒ No
   ☐Yes, if "Yes," ensure the methodology and time line is covered in Section VIII.C.

2. Does the study involve water restriction, food restriction, shock, restraint or other stressors?
   ☐ No
   ☒Yes, if "Yes," ensure that procedures were described in the table in Section VII.B, and that measures taken to assess pain and distress as a result of these procedures were described in Section VII.C

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3. Does the proposed study include the capture, manipulation, observation, housing, tracking, identification, or collection, of any free-living, non-domestic animals?

☒ No

☐ Yes, if "Yes" ensure the methodology and time line is covered in Section VIII.C. Be advised that <u>all necessary permits</u> must be obtained prior to the start of work, and that those permits should be available to the IACUC upon request.

4. Does the proposal include teaching or demonstration as a protocol objective?

☒ No

☐ Yes, if "Yes" ensure that methodology and procedures are covered in

Section VIII.C below.

EMG Implantation   non-invasive ACL injury   force-frequency (neural) / force-length (morphological) / ——euthanized—— / μCT

acclimatize to lab   rest   treadmill acclimatization   8 week exercise interventions for eccentric and concentric groups

0 DAYS   7   11   18 20   76

EMG & kinematic data collections (daily)   EMG & kinematic data collections (weekly)
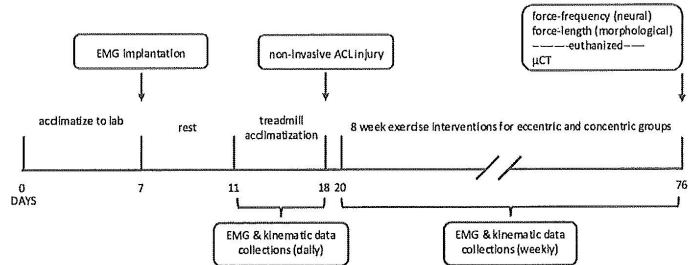
C. <u>Description of Procedures</u>: Provide a complete description of the experimental procedures that will be applied to the animals. Provide sufficient detail to allow the IACUC to discern what is happening to the animals every day the animals are on study; from arrival to endpoint. Reference to an IACUC approved written ACS/ANSCI/IACUC Standard Operating Procedures (SOP), when performing the procedure exactly as stated in the SOP, is acceptable. Include copies of all Department SOP's. Note when surgical procedures are performed but describe them in detail in Appendix G. Tables, timelines and diagrams are helpful. Additional Guidelines for completing this section are listed in the IACUC-1 Instructions Page. DO NOT REPEAT INFORMATION ALREADY GIVEN IN PREVIOUS SECTIONS OR IN APPENDICES. REFER TO PROCEDURES ALREADY DESCRIBED ELSEWHERE BY NAME ONLY.

▶ Aim 1 is a longitudinal study in which weekly measures of knee and ankle kinematics will be collected and serum biomarkers of the inflammatory response will be measured at 1, 3, 7, 14, 28 or 56 days after non-invasive ACL injury. Subsequently, rats will be euthanized and knees will be scanned using μCT to assess PTOA progression, and then dissected to confirm severity of ACL injury. For aim 1, a total of 56 rats (n=28 female and n=28 male) will be used to comprehensively describe our non-invasive ACL injury model. Rats will undergo a one-week acclimatization period in our animal housing facility prior to ACL injury (described above), followed by normal cage activity for 1-56 days. Rats will be euthanized at one of six time points (n=8 rats/time point):1, 3, 7, 14, 28, or 56 days after ACL injury where both knees (unilateral ACL-injured knee and contralateral knee) will be extracted for analysis. Group of 8 (4 males, 4 females) uninjured control rats will also be euthanized at 24 weeks of age. The <u>overall goal</u> of this aim is to establish a non-invasive, clinically-translatable, ACL injury in a rat model and describe the time course of biomechanical alterations, inflammatory response and PTOA progression. This aim will use a separate cohort of rats from aims 2 and 3.

Aims 2 and 3 will use the same cohort of rats in a prospective, randomized controlled laboratory study design in which rats will be randomly assigned to an eccentric (downhill walking), concentric (uphill walking), injury model (no exercise), or control (no injury, no exercise) group (Figure 2). For aims 2 and 3, a total of 96 rats (n=48 males and n=48 females total [n=24 per group; n=12 males, n=12 females) will be used. Aim 2: To investigate the role of exercise on neural factors after non-invasive ACL injury, quadriceps electromyographical (EMG) activity will be measured prior to ACL injury and longitudinally after injury for 8 weeks. On the final day of training the quadriceps force-frequency relationship will be measured. To measure alterations in morphological factors, quadriceps force-length relationship will be measured on the final day of training. Rats will then be euthanized and muscle will also be harvested to assess muscle fiber typing and cross sectional area. Aim 3: Eight weeks after injury, PTOA progression will be assess using μCT to detect changes in microarchitectural parameters of bone and cartilage degradation will be assessed by quantifying differences in cartilage thickness, proteoglycan content and OARSI scores. The <u>overall goal</u> of these aims is to report the effectiveness of eccentric exercise to treat muscle weakness (aim 2) and promote bone and cartilage health after ACL injury (aim 3).

ACL injury:

ACL injury will be induced using a single load of tibial compression. The device consists of two custom-built loading platforms (Figure 3); the top knee stage is rigidly mounted to a linear actuator (DC linear actuator L16-63-12-P, Phidgets, Alberta, CA) that positions the right hindlimb in 30deg of dorsiflexion and 100deg of knee flexion while providing room for anterior subluxation of the tibia relative to the femur; the bottom stage holds the flexed knee and is mounted directly above a load cell (HDM Inc., PW6D, Southfield, MI). Rats will be anesthetized and then the right hindlimb will be subject to single load of tibial compression at a speed of 8mm/s. Anesthesia will be induced using an induction chamber with 5% isoflurane and 1L/min oxygen, and maintained via a nose cone with 1-3% isoflurane and 500mL. ACL injury will be noted by a release of compressive force during injury that will be monitored via our custom program (LabVIEW, National Instruments, Austin, TX). Post-injury, the PI will also perform a Lachman's test to clinically confirm an ACL rupture has occurred.
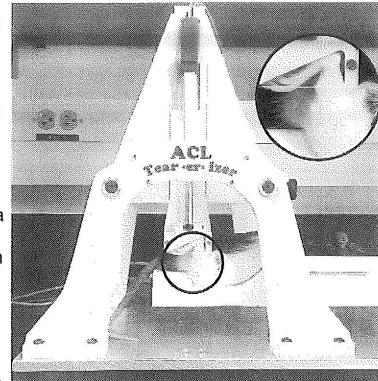
Dr. Lepley has rigorously tested this device over the last year at UConn using post-mortem rats and at a speed of 8 mms/s we have been able to consistently produce an isolated mid-substance ACL injury (n=20 rats). An isolated ACL injury has been confirmed using μCT measures and dissection analyses. Notably, pilot data from Dr. Lepley's post-doc at the University of Kentucky also demonstrates the ability of this device to effectively tear ACLs in a small number of live rats. Analgesics will be withheld as pain medications have been known to influence neural activity and the inflammatory process, two primary outcomes measures of this proposed work. Further, humans after ACL injuries may or may not receive analgesics (i.e. taking analgesics is not the standard of care). Hence withholding analgesics is in-line with the current standard of clinical practice.

Outcome measures:

Inflammatory biomarkers (aim 1). Serial serum measures of inflammatory biomarkers will be collected via the dorsal pedal vein or the tail vein at 1, 3, 7, 14, 28, or 56 days after ACL injury. Rats will be restrained and the dorsal pedal vein or tail vein will be located. The area will then be cleansed with alcohol and the vein will be punctured with a 23G needle if the dorsal pedal vein method is used or a sterile scalpel for the tail prick method. Blood will be collected via a capillary tube and slight pressure will be applied to the area to stop the bleeding.

Hindlimb gait (aims 1 & 2). During level-ground walking (16 m/min) on a motor-driven treadmill, hindlimb kinematic data will be collected using a high-speed digital camera (Figure 4). Kinematics will be collected using retro-reflective skin markers that will be placed on the greater trochanter, lateral femoral condyle, lateral malleolus and fifth metatarsal of the ACL injured hindlimb for the purpose of determining alterations in knee and ankle joint excursions. Knee and ankle kinematics will be calculated using the trajectories of the different joint markers to reconstruct sagittal angular excursions of the knee and ankle joints.
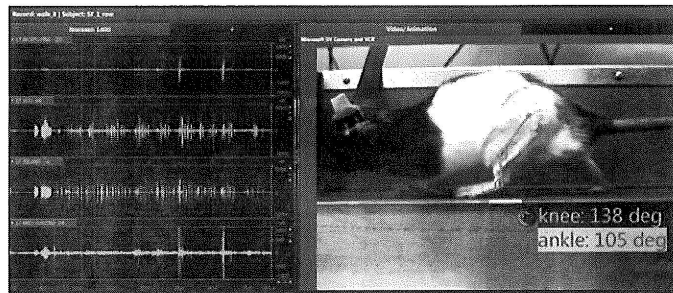
Fine-wire EMG surgical instrumentation (aim 2 - survival surgery). Rats will be instrumented with fine-wire EMG after a one-week period of laboratory acclimatization. Anesthesia will be induced using an induction chamber with 5% isoflurane and 1L/min oxygen, and maintained via a nose cone with 1-3% isoflurane and 500mL oxygen. After reaching the surgical plane of anesthesia, an incision will be made on the anterolateral portion of the left hind limb, and blunt dissection will be used to identify the vastus lateralis muscle. Once the vastus lateralis muscle is identified, custom fabricated, indwelling EMG electrodes constructed of Teflon-coated 10 strand stainless wire (AS-631, Cooner Wire, Chatsworth CA, USA) will be implanted in line with the muscle fibers using a small, curved surgical needle. Wires will be then routed through a subcutaneous tunnel along the spine of the rat to the base of the skull, where a 1cm sagittal plane incision will be made along the coronal suture, just anterior to the lambdoid suture. The top of the skull will then be exposed and cleaned, and four holes will be drilled into the outer dense layer of the skull. Four stainless steel screws (4mm self-taping bone screws, Fine Science Tools, Foster City CA, USA) will be secured into the holes and a 10-pin connector header (Digi-Key Corporation, Thief River Falls MN, USA) will be secured to the top of the skull using layers of dental cement (Excel Formula Dental Material, St. George Technology Inc., Wilmington NC, USA) that will bond to the four screws. The leads of the EMG electrodes will then be soldered to 2 corresponding and opposite pins of the head connector. Following attachment of the EMG leads and one ground

electrode, all exposed pins will be covered with a final layer of dental cement, and the incision will be closed using 4-0 vicryl sutures or with stainless steel wound clips, and rats will be allowed to recover on a heated pad. Immediately post-surgery, rats will also be given Buprenorphine injection (0.02 mg/kg, subcutaneous injection) with additional Buprenorphine injections every 8-14 hours thereafter for 72 hours. Rats will recover for four days. Alternatively, Meloxicam may also be used for 72 hours [3 total doses [dose 1 is administered pre-op], 1-2 mg/kg subcutaneous injection, ACS SOP #2003]. During treadmill walking, raw EMG data (3000 Hz) from the vastus lateralis will be transmitted to a computer via a shielded, multi-conductor that will be attached to the external connector secured to the rat's head and synchronized with hindlimb gait during level-ground walking (Figure 4). EMG data will be acquired from the same step-cycles as the knee and ankle kinematics described above where EMG onset time, phase duration will be calculated.

Nerve cuff surgical instrumentation (aim 2 – non-survival surgery). On the final day of training, rats will be instrumented with femoral nerve cuff electrodes (Figure 2). Rats will be anesthetized (as described above) and a small incision will be made just distal to the inguinal ligament and dissection will be utilized to expose the underlying femoral nerve. A custom-made nerve cuff electrode will then be secured around the femoral nerve and the wires of the nerve cuff will be routed subcutaneously to the base of the skull using the same methods as described above in the EMG implantation section. Rats will be positioned in a stereotactic frame with the ACL hindlimb secured to a custom fabricated instrumented bar that is placed just proximal to the malleolar axis of the tibia. Maximum knee extensor torque will be recorded at multiple angles (75-85°, 95-105°, 115-125°) and voltage output from the bar will be recorded using the data acquisition and playback software (LabVIEW). At each knee angle, maximum knee extensor torque will also be measured at a range of frequencies (single twitch, 10, 50, 100 and 200 Hz) using a Grass S88x stimulator that will evoke knee extension contractions. The average knee extension torque produced over three trials at each frequency and knee angle will be utilized for creating force-frequency (neural outcome) and force-length (morphological outcome) relationships. Immediately after force-frequency (neural outcome) and force-length testing (morphological outcome) is complete, rats will be euthanized.

*Aim 3. All imaging measures will all be conducted after euthanization.

D. **Location of Live Animal Procedures:** Identify, in the table below, where each of the procedures identified above will be performed. Check all that apply, and provide the additional information requested. ONLY INCLUDE AREAS WHERE LIVE ANIMALS WILL BE BROUGHT; DO NOT INCLUDE FIELD WORK:

Type of activity	Building	Room(s)	Duration of stay*
Animal Housing*	ABL	ACS Assigned	It is the goal of the PI to have each batch of rats tested within 12 weeks. This timeline allows for acclimatization and all experimental procedures.
Non-surgical procedures	ABL	ACS Assigned (treadmill walking and ACL rupture)	
Surgical procedures	ABL	ACS Assigned	Estimated time of surgery for EMG implantation is ~1 hr per rat (survival surgery). Fluid replacement via subcutaneous injection will be administered for all procedures greater than 30 minutes.
Recovery Procedures	ABL	ACS Assigned	Post-EMG implantation rats will be monitored for 2 hours. Rats will also be monitored during Buprenorphine or Meloxicam injections as described above.
Terminal procedures, including euthanasia	ABL	ACS Assigned	
Other- describe:			

*If any animals will be maintained outside of the primary housing facility for more than 12 hours, provide location and rationale here
▶ n/a

Section IX: Animal Husbandry and Transportation

A. Animal Housing:

Please select one answer from the following table:

	This section is not applicable because animals will not be housed for any period of time on this protocol (Proceed to Section IX.C)
	I am familiar with the standard husbandry practices (including <u>environmental enrichment</u>) established by the University for the species I am using, and agree that the practices, will meet my research objectives (Proceed to section IX.C)
	The species I am using will require routine care practices not yet identified by University staff (Proceed to Section IX.B)
X	The objectives of my research will require modifications to standard husbandry practices established by the University, e.g. individual housing of social species (Proceed to Section IX.B)

B. <u>Special Husbandry Requirements:</u>

    1. If the handling and care of your species requires husbandry procedures not yet identified by the University staff (i.e., you are using a novel species or strain with unique characteristics that necessitate additional care), either reference or attach an IACUC approved SOP or describe below. Be sure to include information regarding who will be expected to provide the special husbandry (e.g. ACS, lab staff)
      ▶n/a

    2. If your research objectives will require modifications to existing or identified routine husbandry practices (requires single housing, opting out of environmental enrichment, a different cage change frequency, food/ fluid restriction, custom diets, sterilized food, bedding or enclosures etc.), describe and provide scientific rationale below:
      ▶ We haven't experienced this problem in the past (Lepley et al., 2016), but post-EMG instrumentation it is plausible the single housing may be necessary to minimize distress.

      To minimize distress, the use of moistened food, hydrogel cups, and palatable food treats may also be used after ACL injury and EMG electrode placement.

    3. Will any experimental conditions or manipulations have anticipated effects on the normal physiology, anatomy, or behavior of the animals on study?
      ☐No ☒Yes, If "Yes", describe the effects in detail:
          ▶ After ACL injury, atypical hindlimb gait will likely occur.

    4. Will any aspects of the study require animals to be observed more frequently than once a day; or require additional training of the animal care staff to properly identify and monitor experimentally induced conditions?
      ☐No ☒Yes, If "Yes", describe observation requirements (include frequency, special requirements, and if research personnel or ACS will perform; do not include methods required for surgical recovery, as these are covered in detail in *Appendix G*).
          ▶Surgical recovery detailed in Appendix G

C. <u>Animal Tracking</u>
Briefly describe how animals will be tracked throughout the experiment. Such as use of cage cards, ID tags, photographs, censuses, etc. :
      ▶Animals will be tracked via cage cards and ink marker on tails.

D. <u>Animal Transportation</u>

    1. Will animals be transported in accordance with IACUC Policy #AW-02-2012?
      ☒Yes ☐No, If "No", please complete the table below:

	This protocol will require animals to be transported:	Briefly describe transportation methods and any containers being used. In addition please note who will transport the animals (i.e. ACS or protocol personnel):
	This protocol will <u>not</u> require any transportation of animals outside of the housing facility	

	Within a facility but outside of animal housing areas (i.e., from the housing facility to your laboratory)	
	Between facilities on the Storrs campus	
	To other facilities off campus and/or out of state	
	Other not described	

Section X: Endpoint Criteria, Disposition and Euthanasia

A. <u>Endpoints In Study Design</u> *"Endpoint" is defined as the moment at which an animal:*
- *Is euthanized, and its heart and respiratory functions cease*
- *Has its ownership transferred elsewhere, such as sending livestock for sale or slaughter*
- *Is released back into its natural habitat, such as for field studies*
- *The study ends, without animals having been handled*

1. **Experimental Endpoints: What are the identified endpoints in the study design?**
(Experimental endpoints are defined as the point at which scientific aims and objectives have been reached)
There may be more than one endpoint, list all:
▶ For aim 1, a total of 56 rats (n=28 female and n=28 male) will be used to comprehensively describe our non-invasive ACL injury model. Rats will undergo a one-week acclimatization period in our animal housing facility prior to ACL injury, followed by normal cage activity for 1-56 days. Rats will be euthanized at one of six time points (n=8 rats/time point):1, 3, 7, 14, 28, or 56 days after ACL injury where both knees (unilateral ACL-injured knee and contralateral knee) will be extracted for analysis. Group of 8 (4 males, 4 females) uninjured control rats will also be euthanized at 24 weeks of age.

For aims 2 and 3, a total of 96 rats (n=48 males and n=48 females total [n=24 per group; n=12 males, n=12 females) will be used. To investigate the role of exercise on neural factors after non-invasive ACL injury, quadriceps EMG activity will be measured prior to ACL injury and longitudinally after injury for 8 weeks. On the final day of training the quadriceps force-frequency relationship will be measured. To measure alterations in morphological factors, quadriceps force-length relationship will be measured on the final day of training. Rats will then be euthanized (76 days post-arrival to ACS facility, Figure 2) and muscle will also be harvested to assess muscle fiber typing and cross sectional area and PTOA progression.

2. **Humane Endpoints: What animal welfare or behavioral criteria will be used to assess the need to either prematurely remove an animal from the study, or consult with ACS veterinary staff? Examples of criteria could include weight loss, decreased activity, tumor burden, etc. If a field study of free-living animals captured for research, address whether injury from capture process, markers, etc. is likely, and possible responses to observed injury.**
▶ Animals will be inspected for general condition by fur condition, absence of lesions, reduced feed intake, weight loss (20% weight loss, animals will be weighed weekly), and eye discharge. If an animal's general condition is poor, then a veterinary consult will be requested to assess treatment or euthanasia recommendations.

For aim 2, following the surgical implantation of EMG, animals will be weighed every 48 hrs for the first week after surgery and then once a week for the duration of the protocol.

Note: In the event that an animal is found to be in severe pain or distress, all attempts will be made to contact the PI for guidance. However, the veterinarian has authority to use appropriate treatment or control measures, including euthanasia if indicated,

following diagnosis of an animal disease or injury. The veterinarian's authority is exercised with the concurrence of the IACUC and the Institutional Official.

B. <u>Disposition Other than Euthanasia:</u> Will any study animals be alive at the completion of this study?

☒No

☐Yes identify the intended disposition of any live animals at the completion of this study:

This is a field study in which animals are either never handled, or will be released back into their natural environment (wild-caught animals only)	How many animals?
Animals will be transferred to another approved protocol for the same PI	How many animals, and which protocol(s)?
Animals will be transferred to another PI at the University of Connecticut	How many animals, and which PI and protocol?
Animals will be transferred to another PI outside of the University	How many, and where will they be going?
Animals will be transferred from a breeding protocol to a holding, teaching, or research protocol	How many animals?
Other not specified ▶	Describe in detail below ▶

C. <u>Euthanasia/Method:</u> Complete this section to describe euthanasia methods, perfusion and terminal harvest, and carcass disposal procedures being used for this protocol. Note that all methods of euthanasia should be congruent with the AVMA guidelines on euthanasia. IF THIS IS A FIELD STUDY IN WHICH EUTHANASIA IS NOT AN ENDPOINT AND/OR IS PROHIBITED BY THE TERMS OF YOUR PERMIT, SKIP TO SECTION XI.

(Check here)	Chemical Euthanasia Method	Species (indicate all that apply)	Indicate defined study endpoints this method will be used for (IE end of experiment, brain tissue harvest, etc.)	Will be used for non-experimental purposes (IE culling of animals that cannot be used on experiment, humane endpoints, etc.)	Agent and exposure route to be used (IP. IM, SQ, IV, etc.):	Dosage or flow rate:	Other information:
	Overdose of injectable anesthetic						
x	Inhalation of carbon dioxide from a compressed gas cylinder	Rattus, Long Evans	End of experiment	If applicable, humane endpoints	[Inhalation chamber gas CO2 is only acceptable method for this agent]		In accordance with ACS SOP#2006
	Overdose of inhalant anesthetic						
	Overdose of injectable barbituate						
	Other- Indicate here:						

(check here)	Physical/ Combined Euthanasia Method	Species (indicate all that apply)	Indicate defined study endpoints this method will be used for	Will be used for non-experimental purposes (IE culling of animals that cannot	Agent and exposure route to be used (IP.	Dosage or flow rate:	Other information:

			(IE end of experiment, brain tissue harvest, etc.)	be used on experiment, humane endpoints, etc.)	IM, SQ, IV, etc.):		
	Cervical dislocation without anesthesia				N/A	N/A	Justification required
	Cervical dislocation with anesthesia						
	Decapitation without anesthesia				N/A	N/A	Justification required
	Decapitation with anesthesia						
	Exsanguination under anesthesia						
	Perfusion under anesthesia						
	Other- describe:						

1. <u>Justification to use physical euthanasia methods alone:</u> *(complete only if using a physical method as the primary euthanasia method).* The use of physical methods as the sole method for humanely euthanizing animals requires scientific justification. What is/are the reason(s) that anesthetic cannot be used prior to the use of the selected physical method(s)?
▶

2. <u>Verification of Death:</u> How will animal death be verified prior to disposal of animal carcasses? (check all that apply)

x	Thoracotomy under anesthesia	x	Prolonged exposure to CO_2 (>5 minutes)
	Cervical dislocation		Observation of vital signs for 5 minutes
	Rapid freezing (neonatal rats and mice)		Decapitation
x	Exsanguination under anesthesia		Other:

3. <u>Carcass Disposition:</u>

a. Will animal tissues or fluids be potentially contaminated with chemical or biological agents of known or unknown toxicity, reactivity, or infectivity?
☒No ☐Yes, if Yes, you must complete Appendix D

b. Will tissues or fluids be harvested from the euthanized animals?
☐No ☒Yes

c. Will carcasses be disposed of by ACS or Veterinary Pathobiology services?
☒Yes ☐No, if No, describe what will be done with animal carcasses:
▶

D. <u>Euthanasia/Training:</u> Please describe the training program for ensuring the procedures are performed humanely and appropriately:
▶ For CO_2 behavioral cues such as nose twitch and respiratory rate, as well as reaction to application of noxious stimuli (pedal reflexes by toe pinch, and palpebral reflexes) and assessment of jaw tone will be used to monitor the primary

means of euthanasia. Once the behavioral cues are no longer responsive, exsanguination will be performed for assurance of non-revival.

ACS provides training in euthanasia for all new personnel.

<u>Section XI</u>: Occupational Health and Safety Considerations

<u>Hazards Associated With Animal Research</u>: The PI is responsible for ensuring that exposure of humans to physical, chemical, environmental, biological, and radiation hazards is minimized to the greatest extent possible during the course of active research. All people with animal exposure must enroll in the OHS Program for animal handlers. All people with animal exposure are required to complete the necessary EHS training as indicated based on hazard/exposure type. Please review and complete the following:

☒I have read and understand the Occupational Health and Safety Program for Animal Handlers
http://www.ehs.uconn.edu/Biological/ahpr6.pdf

☒I have attached the <u>Workplace Hazard Assessment</u> (WHA) form for my lab/workplace
http://www.ehs.uconn.edu/forms/WHA.php
☒I will submit to EHS an <u>Employee Safety Orientation</u> (ESO) form for each person working under this protocol
http:www.ehs.uconn.edu/forms/ESO.pdf
☒I understand that all animal work can potentially expose personnel to allergens derived from animal fur, feathers, dander or fluids. Each person working under this protocol will either take the EHS Biosafety in Animal Research Training or will be trained by the PI on this topic.

A. <u>Physical risks</u>:

1. Will the nature of the study potentially expose personnel to animal bites, kicks, scratches, punctures, etc.? Ensure that any risks noted below are described in your <u>WHA</u>.
☐No
☒Yes, for rodents or other traditional laboratory species only
☐Yes, for non-traditional species whose use poses minimal physical or biological risk to personnel or animals
☐Yes, for large or non-traditional/ exotic species whose handling may increase risk to personnel.

2. Does the work covered by this protocol increase the likelihood of personnel acquiring injuries due to needle sticks, falls, slips and trips, bruising, or other physical hazards (including risks encountered in the field as a direct result of the research)? Ensure that any risks denoted by a "yes" below are described in your <u>WHA</u>.
☐No
☒Yes

3. Does the work covered by this protocol expose personnel to industrial or occupational hazards, such as loud noise, dangerous machinery, heavy lifting, etc.? Ensure that any risks denoted by a "yes" below are described in your <u>WHA</u>.
☒No
☐Yes

B. <u>Chemical risks</u>:

1. Will the nature of the study potentially expose the <u>PI or research staff</u> to any chemical risks, including but not limited to: formaldehyde or other preservative fluid, cleaning agents, pesticides, poisons, solvents, drugs with known effects, drugs with unknown effects, volatile gases, irritants, potentially explosive substances, etc.
☒No
☐Yes, for chemicals KNOWN NOT TO BE toxic*, chemical carcinogens, reproductive hazards: Ensure that all personnel will complete the annual lab safety training provided through EHS and will read and understand the Chemical Hygiene Plan for the University of CT. PLEASE LIST CHEMICALS BELOW:
▶

☒Yes, for chemicals KNOWN TO BE toxic* but WILL NOT be used in/with live animals: Ensure that all personnel will complete the annual lab safety training provided through EHS and will read and understand the Chemical Hygiene Plan for the University of CT. PLEASE LIST CHEMICALS BELOW:
▶ Acetone, methanol, and ethanol for tissue processing

☐Yes, for chemicals KNOWN TO BE toxic*, chemical carcinogens, reproductive hazards (including but not limited to tamoxifen, BRD-U, etc.) that WILL BE USED IN/WITH LIVE ANIMALS: *Complete and submit Appendix D.*

☐Yes, for experimental compounds: *Complete and submit Appendix D.*

2. Will the nature of the study potentially expose the <u>Animal Care Services staff</u> to any chemical risks, including but not limited to: formaldehyde or other preservative fluid, cleaning agents, pesticides, poisons, solvents, drugs with known effects, drugs with unknown effects, volatile gases, irritants, potentially explosive substances, etc.
☒No
☐Yes, for chemicals KNOWN NOT TO BE toxic*, chemical carcinogens, reproductive hazards: PLEASE LIST BELOW and ensure that all personnel have been provided with the Material Safety Data Sheets for the chemicals to which they may be exposed.
▶

☐Yes, for chemicals KNOWN TO BE toxic*, chemical carcinogens, reproductive hazards (including but not limited to tamoxifen, BRD-U, etc.): *Complete and submit Appendix D.*

☐Yes, for experimental compounds: *Complete and submit Appendix D.*

*A toxic chemical is defined as any chemical with an LD_{50} <500 mg/kg (oral, rats); LD_{50} <1,000 mg/kg (skin, rabbits); or LC_{50} <20,000 mg/m^3 for 1 hr. (inhalation, rats). Source: National Research Council. (2011). *Prudent Practices*. Washington, D.C.: National Academy of Sciences.

C. <u>Biological risks</u>:

1. Will the nature of the proposed study potentially expose personnel or animals to any biological hazards, including but not limited to infectious agents, biological toxins, experimental vaccines, materials of human origin, recombinant DNA, etc. Ensure that any risks denoted by a "yes" below are described in your <u>WHA</u>.

☒No
☐Yes
a. Indicate any required biosafety level requirements here▶ BSL__
b. Complete and submit *Appendix D* section for biological hazards.

You may also be required to submit a protocol/project proposal for review and approval by the Institutional Biosafety Committee (IBC), contact the IACUC office for further clarification

D. <u>Radiation Hazards</u>:

1. Will the nature of the experimental procedures require handling or manipulation of sources of ionizing radiation, including but not limited to x-rays, tomography scans, isotopes, irradiation of cells or animals, lasers, etc.? PLEASE NOTE THAT LICENSURE AND SPECIALIZED TRAINING IS REQUIRED FOR THIS TYPE OF WORK. CONTACT EHS FOR FURTHER GUIDANCE. Ensure that any risks denoted by a "yes" below are described in your <u>WHA</u>.

☒ No
☐Yes Complete and *submit Appendix D.*

E. <u>Regulated Waste</u>:

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☒Yes, for chemicals KNOWN TO BE toxic* but WILL NOT be used in/with live animals: Ensure that all personnel will complete the annual lab safety training provided through EHS and will read and understand the Chemical Hygiene Plan for the University of CT. PLEASE LIST CHEMICALS BELOW:
► Acetone, methanol, and ethanol for tissue processing

☐Yes, for chemicals KNOWN TO BE toxic*, chemical carcinogens, reproductive hazards (including but not limited to tamoxifen, BRD-U, etc.) that WILL BE USED IN/WITH LIVE ANIMALS: *Complete and submit Appendix D.*

☐Yes, for experimental compounds: *Complete and submit Appendix D.*

2. Will the nature of the study potentially expose the <u>Animal Care Services staff</u> to any chemical risks, including but not limited to: formaldehyde or other preservative fluid, cleaning agents, pesticides, poisons, solvents, drugs with known effects, drugs with unknown effects, volatile gases, irritants, potentially explosive substances, etc.
   ☒No
   ☐Yes, for chemicals KNOWN NOT TO BE toxic*, chemical carcinogens, reproductive hazards: PLEASE LIST BELOW and ensure that all personnel have been provided with the Material Safety Data Sheets for the chemicals to which they may be exposed.
   ►

☐Yes, for chemicals KNOWN TO BE toxic*, chemical carcinogens, reproductive hazards (including but not limited to tamoxifen, BRD-U, etc.): *Complete and submit Appendix D.*

☐Yes, for experimental compounds: *Complete and submit Appendix D.*

*A toxic chemical is defined as any chemical with an LD_{50} <500 mg/kg (oral, rats); LD_{50} <1,000 mg/kg (skin, rabbits); or LC_{50} <20,000 mg/m^3 for 1 hr. (inhalation, rats). Source: National Research Council. (2011). *Prudent Practices*. Washington, D.C.: National Academy of Sciences.

C. <u>Biological risks</u>:

1. Will the nature of the proposed study potentially expose personnel or animals to any biological hazards, including but not limited to infectious agents, biological toxins, experimental vaccines, materials of human origin, recombinant DNA, etc. Ensure that any risks denoted by a "yes" below are described in your <u>WHA</u>.

   ☒No
   ☐Yes
       a. Indicate any required biosafety level requirements here► BSL__
       b. Complete and submit *Appendix D* section for biological hazards.

       You may also be required to submit a protocol/project proposal for review and approval by the Institutional Biosafety Committee (IBC), contact the IACUC office for further clarification

D. <u>Radiation Hazards</u>:

1. Will the nature of the experimental procedures require handling or manipulation of sources of ionizing radiation, including but not limited to x-rays, tomography scans, isotopes, irradiation of cells or animals, lasers, etc.? PLEASE NOTE THAT LICENSURE AND SPECIALIZED TRAINING IS REQUIRED FOR THIS TYPE OF WORK. CONTACT EHS FOR FURTHER GUIDANCE. Ensure that any risks denoted by a "yes" below are described in your <u>WHA</u>.

   ☒ No
   ☐Yes Complete and *submit Appendix D*.

E. <u>Regulated Waste</u>:

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1. Will the nature of the proposed study require the disposal of regulated waste of any kind, including bedding, carcasses, regulated medical wastes, etc. that could be contaminated with biological hazards, chemical hazards or radioisotopes?

☒No
☐Yes Complete and *submit Appendix D*

Section XII: Personnel and Training: Please complete Appendix C, listing all personnel, including the PI, who will be assigned to work on this project in its first year. If personnel are added or dropped, resubmit Appendix C as often as necessary to keep it current.

Section XIII: Record-Keeping: Attach copies of, or provide links to, all record forms you plan to use (as defined below):

A. Where will <u>animal husbandry records</u> be maintained for animals on this protocol?

☐ N/A, Animal will not be housed under this protocol
☒ All records will be kept in the ACS facility where the animals are housed.
☐Other- Describe building and location where records can be found:
▶

B. Where will individual <u>animal medical records</u> be maintained?

☐ N/A, Animal will not be housed under this protocol
☒Animals are housed at ACS facility. Individual animal medical records are kept by ACS staff with the animals. Research activities that impact the animals are documented in the records by PI and/or PI's staff.
☐Other- Describe building and location where records can be found:
▶

C. Where will <u>research/experimental records</u> be maintained? Describe & provide building and room:
▶ Research/experimental records will be maintained by the PI in her laboratory (ABL 205) and offices. The PI's laboratory is located in ABL room 205 and her offices are located in 205A ABL and 218 Gampel Pavilion.

D. Where will <u>surgical records</u> be maintained? Describe & provide building and room:

☐ N/A, No surgeries will be performed under this protocol
☒Other- Describe building and location where records can be found:
▶ Research/experimental records will be maintained by the PI in her laboratory (ABL 205) and offices. The PI's laboratory is located in ABL room 205 and her offices are located in 205A ABL and 218 Gampel Pavilion.

<u>Section XIV:</u> Statement of Literature Search and Duplication of Research

<u>Duplication of Research Statement</u>: Federal regulations require that no unnecessary duplication of research be performed. Please complete the following statement, indicating, in your own words, why you feel that the research or teaching goals of this protocol are not unnecessarily duplicative:

As the Principal Investigator responsible for the information contained in this protocol, I have performed the following literature searches, bibliographical searches, and consultation(s) with colleagues in my field of research, on the procedures and objectives contained in this submission:(indicate any that apply)

Check all that apply, *using at least one source,* and supply additional information:

	Literature search was conducted	Which database(s): PubMed and Web of Science	Key words used to search: anterior cruciate ligament OR ACL AND neural OR muscle activity AND muscle morphology AND osteoarthritis OR subchondral AND eccentric exercise OR lengthening exercise OR	Years that were searched: 1980-present	Date the search(s) was/were completed: June 2017
x					

			negative loading AND animal		
	Consultation with in-house colleagues	**Who was consulted?**		**What date was the consultation performed?**	
x	**Consultation with external colleagues**	**Who was consulted?** Dr.Timothy Butterfield		**What date was the consultation performed?** Continuous consultation while writing the grant Spring 2017	Continuous consultation spring/summer 2017
x	**Journals and/or Bibliographical sources were used**	**What titles were used?** • Bigoni, M., Turati, M., Gandolla, M., Sacerdote, P., Piatti, M., Castelnuovo, A., Franchi, S., Gorla, M., Munegato, D., Gaddi, D., 2016. Effects of ACL reconstructive surgery on temporal variations of cytokine levels in synovial fluid. Mediators of Inflammation 2016, 8243601. • Haslauer, C.M., Proffen, B.L., Johnson, V.M., Hill, A., Murray, M.M., 2014. Gene expression of catabolic inflammatory cytokines peak before anabolic inflammatory cytokines after ACL injury in a preclinical model. Journal of Inflammation 11, 34. • Jones, M.D., Tran, C.W., Li, G., Maksymowych, W.P., Zernicke, R.F., Doschak, M.R., 2010. In vivo microfocal computed tomography and micro–magnetic resonance imaging evaluation of antiresorptive and antiinflammatory drugs as preventive treatments of osteoarthritis in the rat. Arthritis & Rheumatism 62, 2726-2735. • Mendias, C.L., Lynch, E.B., Davis, M.E., Enselman, E.R.S., Harning, J.A., DeWolf, P.D., Makki, T.A., Bedi, A., 2013. Changes in Circulating Biomarkers of Muscle Atrophy, Inflammation, and Cartilage Turnover in Patients Undergoing Anterior Cruciate Ligament Reconstruction and Rehabilitation. The American journal of sports medicine 41, 1819-1826. • Wurtzel, C.N., Gumucio, J.P., Grekin, J.A., Khouri, R.K., Russell, A.J., Bedi, A., Mendias, C.L., 2017. Pharmacological inhibition of myostatin protects against skeletal muscle atrophy and weakness after anterior cruciate ligament tear. Journal of Orthopaedic Research Epub ahead of print.	**What year(s) were the publications?** 2010-present	
	Other source(s) were used	**Describe:**			

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I have carefully considered the need to perform the described procedures in live vertebrate animals. I certify , based on my 4 years of experience in the field, careful consideration of the research or teaching objectives, and consideration of the literature review, consultations, and conferences referenced, that the proposed work is <u>not</u> <u>unnecessarily duplicative</u>. I believe this to be true because PTOA is a major cause of lifetime disability, for which no cure currently exists. PTOA is especially problematic following ACL injury, where regardless of treatment, PTOA affects over 50% of ACL injured limbs. Using a rodent model that closely resembles human ACL injury, the proposed studies will elucidate whether eccentric exercise is more effective than concentric exercise at promoting muscle and joint health after ACL injury for the purpose of providing clinicians with evidence-based treatment options for the nearly 300,000 Americans who experience ACL injury each year. Based on the aforementioned, I believe that using Rattus, Long Evans is the most appropriate method for reaching my teaching or research goals.

<u>Section XV:</u>   PI Responsibilities and Signature
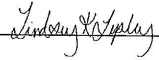
I certify that:

I will comply with IACUC Policy SI-05-2011: "Principle Investigator Responsibilities". A copy of this policy is available at the IACUC website.

The information provided within this application is accurate to the best of my knowledge. I understand that, should I use the project described in this application as the basis for a proposal of intramural or extramural funding, it is my responsibility to ensure that the description of animal use in such funding proposal is identical in principle to that contained in this application.

Principal Investigator:

Name: Lindsey K. Lepley

Signature: _Lindsey K Lepley_

Date: 10/7/17

Student (if student project):

Name:

Signature: _____

Date:

Comments:

<u>Section XVI:</u> Department Head/Chair Signature

I certify that I have reviewed the content of this protocol.

Name: Craig R. Denegar

Signature: _____

Date: 10/9/17

University of Connecticut

Appendix C: Personnel Assigned To Work on Animal Research Protocols
Institutional Animal Care and Use Committee, Office of Research Compliance
Whetten Graduate Center, Rm #214, 438 Whitney Road Ext., Unit 1246 Storrs, CT 06269-1246 860-486-8802

Instructions: This form is a companion form to Section XII of form IACUC-1, "Animal Care and Use Protocol Submission Form". The completion and submission of this form is required for all protocol submissions, and describes:

- The name, background, training, and qualifications of all persons assigned to work on the aforementioned animal research or teaching protocol

Section I: PI (Principal Investigator) and Protocol Information

Principal Investigator (PI): Lindsey K Lepley PhD, ATC
Project Title: Influence of Eccentric Exercise on Muscle and Joint Health Following ACL Injury

Section II: Qualifications of PI

A. **Please include a paragraph summarizing the qualifications of the PI;** including brief description of background and expertise, animal use training, years of experience with laboratory animals, and other relevant University of Connecticut or external training received:

▶ Dr. Lepley is an Assistant Professor in the Department of Kinesiology. Dr. Lepley was appropriately trained by the veterinary staff and Dr. Butterfield at the University of Kentucky as a post-doc to surgically instrument animals for in-vivo protocols to assess changes in neuromuscular function in response to exercise or joint injury. Specifically, Dr. Lepley has been trained to surgically instrument rodents with electromyography and nerve cuffs to examine changes in neural activity in response to injury. She also has experience in conducting exercise interventions using motor-drive treadmills with rodents. Dr. Lepley has 4 years of laboratory animal experience and 7 of experience working with humans. Dr. Lepley's research line seeks to identify the neuromuscular consequences of traumatic joint injury and to identify therapeutic approaches capable of combating neuromuscular dysfunction.

Section III: Project Personnel

A. **Please complete the chart on the following page** to describe the qualifications and training of each individual who will be assigned a role for work on this project (excluding PI and unlisted students below)

Section IV: Unlisted Students and Personnel

A. **Will any students or other personnel not listed below in Section VI be assigned to work with animals on this protocol?**

☒No ☐Yes, if Yes, qualify below

1. Who will supervise or oversee these individuals?
   ▶

2. Describe the training these individuals will receive prior to working on this protocol:

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▷

3. Describe the types of activities these individuals will be engaged in with regards to animal procedures:

▷

<u>Section V:</u> Occupational Safety and Health Registration

A. <u>Have all personnel listed in Section V been enrolled in the University's Occupational Health and Safety (OHS) Program for Animal Handlers</u>, by completing and submitting the appropriate forms from the EHS web site?

☒Yes ☐No (Note that individuals will not be approved to work under this protocol until all required OHS documentation is complete)

Special note regarding ethics in research training:

The Office of Research Compliance would like to remind Principal Investigators that effective January 4, 2010, changes in federal regulations mandate that the University of Connecticut must certify that a plan is in place for providing the appropriate training and oversight in the responsible and ethical conduct of research (RCR) to all technicians, undergraduate students, graduate students, and postdoctoral researchers who participate <u>in federally funded research (renewals or new applications)</u>. It is not yet required of faculty members; however they are encouraged to take the course. The RCR curriculum will be offered in a variety of formats (in-person and on-line) and must be completed during the course of the individual's involvement in the federally funded project. <u>It is NOT required that the training be completed prior to involvement in a project</u>. More information about RCR is available at http://vprge.uconn.edu/rcr.cfm.

This training is not required for IACUC review, but is required for the procurement of federal funds for research.

Section VI: Listed Protocol Personnel:

Name of individual (last, first, MI)	Status (student, etc.)	Procedures this individual will perform	Species this individual will work with	# of years and type of experience working with this species	Type of experience working with these procedures	If this person has worked w/ species or procedure <1 year, describe training that will be provided prior to beginning work on this protocol	Who will provide this training?	List date and type of most recent IACUC training (Initial classroom session, on-line, one-on-one, etc.)	Describe any other relevant training and experience
Davi, Steven, M	PhD student	Treadmill walking and euthanasia (initially). After appropriate training by the veterinary staff and Dr. Lepley, Steven will also assist with surgically instrumenting animals for in-vivo protocols to assess changes in neuromuscular function in response to exercise or joint injury.	Long Evans rats	1	Assisted Dr. Lepley with treadmill walking and euthanasia procedures AYs 15-17	n/a	Dr. Lepley and the veterinary staff	Fall 2016 – IACUC training 10/26/16 – euthanasia training	

University of Connecticut

Appendix E: Considerations of Alternatives to Painful or Distressful Procedures in Animal Research Protocols
Institutional Animal Care and Use Committee, Office of Research Compliance
Whetten Graduate Center, Rm #214, 438 Whitney Road Ext., Unit 1246 Storrs, CT 06269-1246 860-486-8802

Instructions: This form is a companion form to Section VII of form IACUC-1, "Animal Care and Use Protocol Submission Form". The completion and submission of this form is required if your protocol submission contains:

- Live vertebrate animal research procedures classified as <u>USDA Pain Category D or E</u>

Section I: PI (Principal Investigator) and Protocol Information

Principal Investigator (PI) Lindsey K Lepley PhD ATC
Project Title: Influence of Eccentric Exercise on Muscle and Joint Health Following ACL Injury

Section II: Background

    A.   <u>USDA Policy #11</u> provides guidance on the use of potentially painful or distressful procedures in animal care and use

    B.   <u>USDA policy # 12</u> requires that procedures involving live animals will be designed in such a way as to minimize discomfort, distress, and/or pain to that which is absolutely necessary to meet research goals, and requires a written narrative of the consideration of alternatives to painful or distressful procedures

    C.   The *Guide for the Care and Use of Laboratory Animals* requires that a veterinary consultation *must* occur when pain or distress is beyond the level anticipated in the protocol description or when interventional control is not possible

Section III: Search for Alternatives

    A.   For *each* protocol procedure that is likely to produce pain and/or distress, please complete the following table for any literature search that was conducted. At least one database or other source must be referenced:

Name of procedure	Date literature search was performed (month/year)	Years covered by search	Database(s) searched	All Keywords Used to search (or search strategy) (must contain the following search terms: "alternative", "[procedure]", and "[species]"	Number of "hits" returned
Non-invasive ACL Injury	11/2016, 3/2017, 6/2017	1980-present	PubMed and Web of Science	rat AND ACL OR anterior cruciate ligament OR AND alternative AND species	0

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				rat AND	
EMG instrumentation	11/2016, 3/2017, 6/2017	1980-present	PubMed and Web of Science	electromyographical OR EMG AND quadriceps AND alternative AND species	27
Femoral nerve cuff	11/2017	1980-present	PubMed and Web of Science	rat AND nerve cuff AND quadriceps AND alternative AND species	0

B. It is also acceptable to utilize current material from other resources. If you used any of the following to evaluate procedures on your protocol, please supply the following information:

Name of Procedure:	Method used to search for alternatives:	Source utilized:	Summary of Topics, searches, strategies, discussions, etc.	Date
	Consultation with in-house colleagues	Who was consulted?		What date was the consultation performed?
x	Consultation with external colleagues/ experts	Who was consulted? Timothy A Butterfield, University of Kentucky	Discussion of current scientific literature and key gaps these proposed experiments will fill/ necessity of animal model	What date was the consultation performed? Continuous conversations spring/summer 2017
	Journals and/or Bibliographical sources were utilized	What titles were utilized?		What year(s) were the publications?
	Other source(s) were utilized	Describe:		

C. Evidence of Veterinary Consult: (Provide the date that you or your staff consulted (phone, email or in person is acceptable) with a member of the ACS veterinary staff to discuss Category D or E procedures)

▶ Dr. Lepley met with Dr. Ramaswamy Chidambaram on 10/11/17 to discuss the procedures listed in this IACUC.

Section IV: Written Narrative

A. For *each* search identified in Section III above, please draft a narrative statement that summarizes the relevance of the "hits" your search provided to the context of the objectives and goals of the study. You must include information in your narrative as to why any alternatives found cannot be used for this protocol:
▶ We specifically require animal models for the non-invasive ACL injury because wish to measure multiple tissues and muscle function in freely moving, unconstrained, ambulating animals with hopes of translating our results to the human population (e.g. complexity of the processes being studied cannot be replicated, duplicated, or modeled in simpler living systems or other invertebrates). Rat models have been used extensively to study the effect of ACL injury on OA. To date, the most widely used model of ACL injury is ACL transection,(Galois et al., 2004; Hashimoto et al., 2002; Kadonishi et al., 2012; Martin and Buckwalter, 2006; Yamaguchi et al., 2013) which is an acute model that surgically destabilizes the joint. Though practical from a methodological standpoint, the surgical transection of the ACL is not a viable model to study the neuromuscular maladaptations that lead to OA development after ACL injury for two primary reasons. First, violation of the joint capsule creates a cascade of negative neuromuscular events that confounds the already altered neural signaling that is present due to the loss of the ACL.(Lepley et al., 2015; Lepley and Palmieri-Smith, 2016; Rice and McNair, 2010) Second, opening the joint capsule without reinstitution of anterior-posterior stability achieved through surgical reconstruction creates a model of injury that is not present in humans. Non-invasive ACL injury models are required to circumvent these issues. Our recent pilot data shows that we can successfully produce an isolated in-vivo ACL rupture through progressive tibial overload (described in

IACUC_1 document). This cutting edge work is in-line with others, as a recently published article (Oct 2016)(Ramme et al., 2016) has successfully shown that post-traumatic OA can be induced in rats using similar methodology and OA timelines that we proposed here (i.e. 8 weeks is sufficient to detect OA onset). Our search strategy yielded no suitable alternatives to this procedure (Category E). For aim 1, 48 rats will undergo the non-invasive ACL injury (86%) and 8 rats will serve as healthy controls (no injury, 14%). For aims 2 and 3, 72 rats will undergo the non-invasive ACL injury (75%) and 24 rats will serve as healthy controls (no injury, 25%). Analgesics will be withheld after ACL injury, as pain medications have been known to influence neural activity and the inflammatory process, two primary outcomes measures of this proposed work. Further, humans after ACL injuries may or may not receive analgesics (i.e. taking analgesics is not the standard of care). Hence withholding analgesics is in-line with the current standard of clinical practice.

We also wish to examine the specific alterations in neural excitability that contribute to OA onset after ACL injury. Hence EMG instrumentation will allow us to discretely observe important neural alterations in the quadriceps muscle that manifest after injury using a prospective study design. Notably, our search strategy utilized above yielded no suitable alternatives to this procedure and 100% of the rats involved in aims 2 of this proposed work (n=96 rats) will be exposed to the category D procedures.

Lastly we also plan to monitor changes in the quadriceps force-frequency relationship using a femoral nerve cuff. The electrical stimulation of the muscle will allow us to gain additional insight into the underlying physiological changes that regulate function after ACL injury. No suitable alternatives to this procedure is available and 100% of the rats involved in aims 2 of this proposed work (n=96 rats) will be exposed to this category D procedure.

University of Connecticut

Appendix G: Use of Surgery in Animal Research Protocols
Institutional Animal Care and Use Committee, Office of Research Compliance
Whetten Graduate Center, Rm #214, 438 Whitney Road Ext., Unit 1246 Storrs, CT 06269-1246 860-486-8802

Instructions: This form is a companion form to Section VIII.A.6 of form IACUC-1, "Animal Care and Use Protocol Submission Form". The completion and submission of this form is required if your protocol submission contains:

- The cutting, abrading, suturing, laser or otherwise physical alteration of body tissues and organs of live vertebrate animals.

- Survival surgical procedures, i.e. the animal must be expected to regain consciousness after the procedure is performed.

- Non-survival surgical procedures, i.e. the animal will not regain consciousness following the procedures: FOR NON-SURVIVAL PROCEDURES COMPLETE THIS APPENDIX THROUGH **SECTION III** ONLY.

NOV 1 5 2017

Section I: PI (Principal Investigator) and Protocol Information

Principal Investigator (PI): Lindsey K Lepley PhD ATC
Project Title: Influence of Eccentric Exercise on Muscle and Joint Health Following ACL Injury

Section II: Providers of Care: Who will be performing the surgical procedures described in this protocol? Check all that apply:

	Animal Care Services (ACS) Staff
	Agricultural animal care staff
x	Principal Investigator (PI) and/or research staff
x	Students under the direction/ instruction of the PI
	Other (describe)

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<u>Section III:</u> Identification of NON-SURVIVAL Surgical Procedures

    A.   Describe non-survival surgery below, including methods of anesthesia, checking for depth of anesthesia, and surgical preparation and procedures. (n.b., the *Guide 2011* states that for non-survival surgeries, at a minimum, the surgical site should be clipped, the surgeon should wear gloves and the instruments and surrounding areas should be clean).

➤ All rats will be instrumented with femoral nerve cuff electrodes. Rats will be anesthetized and a small incision will be made just distal to the inguinal ligament and dissection will be utilized to expose the underlying femoral nerve. Behavioral cues such as nose twitch and respiratory rate, as well as reaction to application of noxious stimuli (pedal reflexes by toe pinch, and palpebral reflexes) and assessment of jaw tone will be used to monitor the depth of anesthesia. A custom-made nerve cuff electrode will then be secured around the femoral nerve and the wires of the nerve cuff will be routed subcutaneously to the base of the skull using the same methods as described above in the EMG implantation section. Immediately after force-length and force-frequency testing is complete, rats will be euthanized.
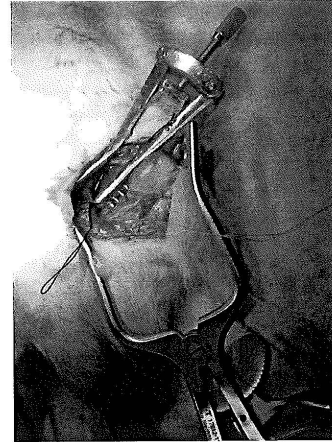
<u>Section IV:</u> Identification of SURVIVAL Surgical Procedures

    A.   <u>Minor surgical Procedures:</u> Minor surgical procedures are surgical procedures that *do not expose a body cavity AND that cause minimal or no physical impairment*

        1.   Will any minor surgical procedures be performed in this protocol?

           ☒No ☐Yes (qualify below)

           a.   Identify all minor surgical procedures used in this protocol:
              ➤

           b.   Which species will receive these procedures?
              ➤

           c.   What is the purpose of the procedures identified (briefly describe in layman's terms)?
              ➤

    B.   <u>Major surgical procedures:</u> Major surgical procedures are surgical procedures that *expose a body cavity AND/OR cause substantial physical or physiological impairment*

        1.   Will any major surgical procedures be performed in this protocol?

           ☐No ☒Yes (qualify below)

           a.   Identify all major surgical procedures used in this protocol:
              ▶ EMG instrumentation

           b.   Which species will receive these procedures?
              ▶ Rattus, Long Evans

           c.   What is the purpose of the procedures identified (briefly describe in layman's terms)?
              ▶ To measure neural activity of the quadriceps muscles during level-ground treadmill walking.

Section V: Surgical Preparation: Answer the following questions related to all survival surgery procedures identified in Section IV:

A. **Preparation of surgical location:**

1. Where will the surgery or surgeries be performed? Specific location(s):
   ▶ ACS assigned room

2. Is this location a dedicated surgical suite? ☒Yes ☐No

3. How will this location be prepped for the surgery (surfaces cleaned, disinfected, or sanitized; surfaces reorganized to accommodate surgery, dedicated hood prepped with UV light, etc.):
   ▶ Yes, we plan to use the ABL surgical room.

4. Animal preparation

   a. Will animal preparation require fasting or fluid restriction? ☒No ☐Yes How long?
      ▶

   b. Will animal preparation include use of a pre-anesthetic or early administration of analgesia? ☐No ☒Yes
      A single dose of Meloxicam may also be used (1-2 mg/kg subcutaneous injection, ACS SOP #2003) before surgery.

   c. Are any additional animal preparations necessary?
      ▶ Yes, ophthalmic ointment will be administered in eyes of all anesthetized animals.

5. Surgical instrument preparation

   a. How will surgical instruments be prepped for surgery?
      ▶ The procedure will be performed in an aseptic area over a heated pad using autoclave–sterilized surgical instruments. The surgeon will wear a mask, sterile gloves and a clean lab coat at all times. Instruments will be kept in a glass bead sterilizer.

   b. How will sterility or asepsis of surgical instruments be maintained throughout the course of the surgery?
      ▶ The surgeon will wear a mask, sterile gloves and a clean lab coat at all times. Instruments will be kept in a glass bead sterilizer.

6. Surgical site preparation:

   a. How will the surgical site be prepped for surgery?
      ▶ The incision sites on the anteromedial aspect of the right knee and head will be shaved and cleaned using three alternating cycles of scrub/ethanol, with a final swipe of Betadine.

<u>Section VI:</u> Conduction of Surgery

A. <u>Anesthesia:</u> Complete the following questions regarding the use of anesthesia for surgical procedures described in this protocol.

1. Will any surgical procedures be performed without anesthesia?

    ☒No
    ☐Yes, if Yes, justification MUST be provided below:

    ▶

2. Please complete the following regarding any primary and secondary surgical anesthesia that will be used for this protocol:

Species	Surgical Procedure	Anesthetic used	General or local anesthetic?	Dose	Route of administration	Timing of administration (pre-surgery, during surgery, etc.)
Rattus, Long Evans	EMG implantation	Isoflurane	General	1L/min oxygen, and maintained via a nose cone with 1-3% isoflurane and 500mL	Nose cone	pre-surgery and during surgery

3. For any general anesthetics listed above, please indicate how depth of anesthesia will be monitored (check all that apply- at least 2 methods must be checked for surgery that requires a surgical plane. One method is acceptable for surgery requiring the numbing of an area)

	Blood pressure/ EKG readings	x	Respiratory rate
	Corneal reflexes	x	Tail/toe pinch (pedal reflexes)
	EEG		Other: specify
	Heart Rate		Other: specify
	Pinching or pricking of incision site (local anesthesia only)		

B. <u>NMB: Neuromuscular Blocking Agents</u>

1. Will any neuromuscular blocking agents, muscle relaxants, or paralytic drugs be used in this protocol? ☒No
    ☐Yes (qualify below)

    a. What species will receive the agent(s)?
        ▶

    b. What drug and dosage?
        ▶

    c. How will respiration and anesthesia depth be monitored while animals are under the influence of these agents?
        ▶

C. <u>Surgical techniques</u>

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1. Describe, in detail, the procedures for any minor surgical techniques identified in Section IV, or provide copy of SOP (or reference to IACUC approved SOP#). If more than one species is used in the protocol, indicate which one(s) are undergoing the procedure:
   ►n/a

2. Describe, in detail, the procedures for any major surgical techniques identified in Section IV, or provide copy of SOP (or reference to IACUC approved SOP#). If more than one species is used in the protocol, indicate which one(s) are undergoing the procedure:

► Rats will be instrumented with fine-wire EMG. After reaching the surgical plane of anesthesia, an incision will be made on the anterolateral portion of the left hind limb, and blunt dissection will be used to identify the vastus lateralis muscle. Once the vastus lateralis muscle is identified, custom fabricated, indwelling EMG electrodes constructed of Teflon-coated 10 strand stainless wire (AS-631, Cooner Wire, Chatsworth CA, USA) will be implanted in line with the muscle fibers using a small, curved surgical needle. Wires will be then routed through a subcutaneous tunnel along the spine of the rat to the base of the skull, where a 1cm sagittal plane incision will be made along the coronal suture, just anterior to the lambdoid suture. The top of the skull will then be exposed and cleaned, and four holes will be drilled into the outer dense layer of the skull. Four stainless steel screws (4mm self-taping bone screws, Fine Science Tools, Foster City CA, USA) will be secured into the holes and a 10-pin connector header (Digi-Key Corporation, Thief River Falls MN, USA) will be secured to the top of the skull using layers of dental cement (Excel Formula Dental Material, St. George Technology Inc., Wilmington NC, USA) that will bond to the four screws. The leads of the EMG electrodes will then be soldered to 2 corresponding and opposite pins of the head connector. Following attachment of the EMG leads and one ground electrode, all exposed pins will be covered with a final layer of dental cement, and the incision will be closed using 4-0 vicryl sutures or with stainless steel wound clips, and rats will be allowed to recover on a heated pad. Immediately post-surgery, rats will also be given Buprenorphine injection (0.02 mg/kg, subcutaneous injection) with additional Buprenorphine injections every 8-14 hours thereafter for 72 hours. Rats will recover for four days. Alternatively, Meloxicam may also be used for 72 hours [3 total doses [dose 1 is administered pre-op], 1-2 mg/kg subcutaneous injection, ACS SOP #2003].

Estimated time of surgery for EMG implantation is ~1 hr per rat (survival surgery). Fluid replacement via subcutaneous injection will be administered for all procedures greater than 30 minutes.

3. Describe any types of sutures, wound clips, staples, glue, or other tissue closure method that will be used:
   ► Incision will be closed using 4-0 vicryl sutures or with stainless steel wound clips (to be removed after 10-14 days). Dr. Lepley has experience in using both of these closure methods (Lepley et al., 2016).

4. How will bleeding be controlled during surgery?
   ► Care will be taken to avoid any large vessels. In our experience, very minimal blood loss occurs during this type of surgical procedure (Lepley et al., 2016).

Section VII: Medications

A. <u>Withholding Analgesia:</u> Complete the following regarding the use of analgesia for this protocol:

1. Will any animals have analgesic use withheld for surgical procedures?

   ☒No ☐Yes, if Yes:

   a. What species and procedures?
      ▶

   b. What is the justification for withholding analgesia?
      ▶

   c. What non-analgesic measures will be taken to alleviate pain or discomfort?
      ▶

B. Administration of medications:

   1. Please complete the following table regarding medications that will be administered to animals due to surgical procedures for this protocol (examples include analgesics, antibiotics, anti-rejection drugs, fluids, etc.):

Species	Surgical Procedure	Medication used	Dose	Route of administration	Timing of administration (pre-surgery, during surgery, post-surgical, peri-surgical)	Frequency and duration of reapplication
Rattus, Long Evans	EMG instrumentation	Buprenorphine	0.2mg/kg	Subcutaneous	Every 8-14 hours after surgery for the first 72 hours	Every 8-14 hours after surgery for the first 72 hours
Rattus, Long Evans	EMG instrumentation	Meloxicam	1-2 mg/kg	Subcutaneous	Pre-surgery and then once every 24 hours for the first 72 hours	Pre-surgery and then once every 24 hours for the first 72 hours
Rattus, Long Evans	EMG instrumentation	Ophthalmic ointment will be administered in eyes of all anesthetized rats		Topical	During surgery	

   3. If medication other than analgesia or antibiotic, please describe its purpose and function:
      ▶n/a

Section VIII: Recovery

   A. Post-operative monitoring

      1. Who will provide immediate post-operative care and observation to the animals?
      ▶ Dr. Lepley

      2. Will animals be provided a warming blanket or other heat source to aid recovery from anesthesia?
      ▶ Yes, a heated pad.

      3. How often will animals be checked, and by whom, prior to fully awakening?

⊢ Every 8-14 hours after surgery for the first 72 hours if Buprenorphine is used. If Meloxicam is used, rats will be monitored every 24 hours for the first 72 hours.

4. **Will animals require any specialized post-operative care? Describe:**
⊢ Buprenorphine or Meloxicam procedures described above. We may need to consider single housing. This has not been a problem in the past, but single housing may help to reduce distress immediately post-surgery. To minimize distress, the use of moistened food, hydrogel cups, and palatable food treats may also be used after EMG electrode placement.

5. **Where will post-operative recovery occur?**
⊢ ACS assigned room

B. Return to regular housing

1. **What are the criteria for determining an animal is ready to be returned to a regular housing facility (or otherwise released back into its environment)?** ▶ Rats will be taken off the isoflurane and allowed to recover on a heated pad in their cage. Rats will be observed continuously until ambulatory and then they will be checked every 15 min for 2 hours. Rats will then be returned to regular housing.

2. **Will animal require any housing or environmental modifications upon its return? (e.g. single housing, dietary supplement, food placed on floor of cage, etc.) Describe:**
▶ Potential considerations for single housing in the acute post-operative stages.

3. **Will animal require sutures or staples to be removed?**
☐No ☒Yes, if Yes: When, and by whom: **Dr. Lindsey Lepley**
▶ If sutures are used, we will use vicryl, an absorbable suture. If stainless steel wound clips are used, clips will be removed after 10-14 days.

C. Surgical Impairment

1. **Describe the anatomical and/or physiological effects the surgery is expected to have on the animals:**
▶ In our experience, the surgical procedure has a minimal physiological effect on the animal. The most notable side effect is lethargy for the first 48 hours. To ensure animal safety post-surgery, rats will be monitored for bleeding, lethargy, loss of grooming behavior, eye discharge, wound infection (drainage, swelling, redness, pain), and weight loss. If an animal loses more than 20% of its preoperative bodyweight (animals to be weighed every 48 hrs for the first week after surgery, then once a week for the duration of the experimental procedure), then a veterinary consult will be requested to assess treatment or euthanasia recommendations.

D. Post-surgical maintenance of animals

1. **Will the effects described above require any long-term maintenance or monitoring of the animals' condition, or will procedures decrease the animal's normal lifespan?**
☒No ☐Yes, if Yes, describe:
▶

E. Surgical Complications

1. **What are the anticipated and potential surgical complications that may be seen with the surgical procedures outlined in this protocol, and what is the plan for dealing with these complications should they arise?**
▶ Rats will be monitored for bleeding, lethargy, loss of grooming behavior, reduced food intake, eye discharge, wound infection (drainage, swelling, redness, pain), and weight loss. If an animal loses more than 10% of its

preoperative bodyweight, then a veterinary consult will be requested to assess treatment or euthanasia recommendations.

2. **What is the expected animal attrition due to surgical complications for this procedure when it is performed by a skilled individual? Ensure that attrition has been considered in the numbers justification section of the main protocol form.**
▶ Animal attrition is limited with this minor surgical procedure. Our power analysis has taken potential attrition into effect by requesting an additional 20% of animals.

3. **Can any of the surgical procedures utilized by this protocol be described as unfamiliar, novel, or infrequently used [by personnel in your laboratory]?**
☒No ☐Yes, if Yes, briefly describe training that will be provided to acclimate research associates:
▶

Section IX: Multiple Major Survival Surgery

A. **Will any animals identified in this document be receiving a second (or more) major survival surgery during the course of this protocol?**
☒No ☐Yes, if "Yes":

1. What is the nature of the two (or more) major survival surgeries?
▶

2. Why are multiple surgeries necessary for meeting the research goals and objectives? (Scientific or animal welfare-related justification is required for this response)
▶

Appendix B: Data Collection Form (Chapter 3)

L Lepley Rodent Anesthesia/Surgery/Post-Operative Record

PI		Protocol # AN		
Personnel		Circle	Survival Surgery	Non-Survival Surgery
Date		Species		
Procedure name		Experimental agents administered		
Anesthetics used		Dosage /Volume admin	(% or mg/kg; mL)	Route
Analgesics used	(1) (2) (3)	Dosage /Volume admin	(1) (2) (3)	(1) (2) (3)

❏ Ophthalmic ointment administered in eyes of all anesthetized animals
❏ Heat provided during surgery and recovery
❏ Pedal reflex (toe pinch- both feet) checked periodically during surgery to evaluate depth of anesthesia

Circle Animal ID Cage ID					
Body weight					
Anesthesia start time					
Analgesics Name	Time administered				
(1)					
(2)					
(3)					
Other agents Name	Time and dosage/volume administered				
A)					
B)					
C)					
D)					
Anesthesia end time					
Complications? Y/N					

Note complications here and on back of page if needed:
_____

POST-OPERATIVE

Recording of post-operative observations is required if the protocol states to give analgesics "if/as needed" or "PRN".
- Record date and time of post-op observation
- Once post-surgical pain assessment has concluded, all other monitoring that is part of the protocol will continue as approved

Animal or Cage ID #					
Date/Time					
Analgesic details					
Date/Time					
Analgesic details					
Date/Time					
Analgesic details					

Appendix C: Data Collection Forms and Surveys (Chapters 4 and 5)

Subject ID: ⬚⬚⬚⬚⬚⬚   **Date:** ⬚⬚ / ⬚⬚ / ⬚⬚
                                      Month   Day   Year

Demographic Form

Instructions: Below is a list of questions about your demographics and health history. Please answer the following questions as completely as possible.

1. What is your sex? (circle all that apply)
   a. male
   b. female
   c. intersex

2. How do you identify? (circle all that apply)
   a. man
   b. woman
   c. non-binary
   d. prefer to self-describe: _____
   e. prefer not to identify

3. Have you been previously diagnosed with radiographic or symptomatic knee osteoarthritis?
   a. yes
   b. no
   c. n/a

4. Date of ACL injury (if exact date is unknown, please provide an approximate date):
   a. date:_____
   b. n/a

5. Date of ACL surgery (if exact date is unknown, please provide an approximate date):
   a. date:_____
   b. n/a

6. Which is your ACL injured limb?
   a. right
   b. left
   c. n/a

7. What ACL graft type do you have?
   a. patellar tendon (aka bone-patellar tendon bone)
   b. hamstring (aka semitendinous gracilis)
   c. quadriceps tendon
   d. tibialis anterior
   e. other: _____
   f. n/a

PI: Garcia, IRB Number: HUM00169174, Protocol Title: A Systems Based Approach to Understand Factors Affecting Knee Articular Cartilage

Subject Initials [ ][ ]   **Subject ID** [ ][ ][ ][ ]   **Testing day** [ ][ ] / [ ][ ] / [ ][ ]
                                                                               Month   Day   Year

Pre-Screening Form

Subject U of M Record Number: [ ][ ][ ][ ][ ][ ]

First Name:	
Middle Name (or initial):	
Last Name:	
Mailing Address:	
Phone Number:	
Email:	

Birthday: [ ][ ] / [ ][ ] / [ ][ ]   **Height (m):** _____   **Weight (kg):** _____
               Month   Day   Year

Instructions: Below if a list of questions about your health history. In order to meet pre-screening criteria, it is important for the research team to know if you have ever been diagnosed or experienced one of the following:

1) Do you have a previous history of ACL injury or knee surgery other than your current ACL reconstruction?

2) Does your current ACL reconstruction use an allograft (e.g., donated tissue) graft type?

3) Have you suffered a lower extremity injury within the past 6 months, other than your current ACL reconstruction?

4) Have you been previously diagnosed with radiographic or symptomatic knee osteoarthritis?

5) Do you have a history of untreated diabetes?

6) If you are female, are you currently pregnant?

Please check one of the following boxes:

[ ] Yes, I have been diagnosed or experienced one of the above-mentioned items

[ ] No, I have not been diagnosed or experienced one of the above-mentioned items

Signature of staff completing screening: _____   **Date:**_____

2000 IKDC SUBJECTIVE KNEE EVALUATION FORM

Your Full Name_____

Today's Date: _____/_____/_____   Date of Injury: _____/_____/_____
           Day   Month   Year   Day   Month   Year

SYMPTOMS*:
*Grade symptoms at the highest activity level at which you think you could function without significant symptoms, even if you are not actually performing activities at this level.

1. What is the highest level of activity that you can perform without significant knee pain?

    4 ❑ Very strenuous activities like jumping or pivoting as in basketball or soccer
    3 ❑ Strenuous activities like heavy physical work, skiing or tennis
    2 ❑ Moderate activities like moderate physical work, running or jogging
    1 ❑ Light activities like walking, housework or yard work
    0 ❑ Unable to perform any of the above activities due to knee pain

2. During the past 4 weeks, or since your injury, how often have you had pain?

	0	1	2	3	4	5	6	7	8	9	10	
Never	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑	Constant

3. If you have pain, how severe is it?

	0	1	2	3	4	5	6	7	8	9	10	
No pain	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑	Worst pain imaginable

4. During the past 4 weeks, or since your injury, how stiff or swollen was your knee?

    4 ❑ Not at all
    3 ❑ Mildly
    2 ❑ Moderately
    1 ❑ Very
    0 ❑ Extremely

5. What is the highest level of activity you can perform without significant swelling in your knee?

    4 ❑ Very strenuous activities like jumping or pivoting as in basketball or soccer
    3 ❑ Strenuous activities like heavy physical work, skiing or tennis
    2 ❑ Moderate activities like moderate physical work, running or jogging
    1 ❑ Light activities like walking, housework, or yard work
    0 ❑ Unable to perform any of the above activities due to knee swelling

6. During the past 4 weeks, or since your injury, did your knee lock or catch?

    0 ❑ Yes   1 ❑ No

7. What is the highest level of activity you can perform without significant giving way in your knee?
    4 ❑ Very strenuous activities like jumping or pivoting as in basketball or soccer
    3 ❑ Strenuous activities like heavy physical work, skiing or tennis
    2 ❑ Moderate activities like moderate physical work, running or jogging
    1 ❑ Light activities like walking, housework or yard work
    0 ❑ Unable to perform any of the above activities due to giving way of the knee

SPORTS ACTIVITIES:

8. What is the highest level of activity you can participate in on a regular basis?

    4 ☐ Very strenuous activities like jumping or pivoting as in basketball or soccer
    3 ☐ Strenuous activities like heavy physical work, skiing or tennis
    2 ☐ Moderate activities like moderate physical work, running or jogging
    1 ☐ Light activities like walking, housework or yard work
    0 ☐ Unable to perform any of the above activities due to knee

9. How does your knee affect your ability to:

		Not difficult at all	Minimally difficult	Moderately Difficult	Extremely difficult	Unable to do
a.	Go up stairs	4 ☐	3 ☐	2 ☐	1 ☐	0 ☐
b.	Go down stairs	4 ☐	3 ☐	2 ☐	1 ☐	0 ☐
c.	Kneel on the front of your knee	4 ☐	3 ☐	2 ☐	1 ☐	0 ☐
d.	Squat	4 ☐	3 ☐	2 ☐	1 ☐	0 ☐
e.	Sit with your knee bent	4 ☐	3 ☐	2 ☐	1 ☐	0 ☐
f.	Rise from a chair	4 ☐	3 ☐	2 ☐	1 ☐	0 ☐
g.	Run straight ahead	4 ☐	3 ☐	2 ☐	1 ☐	0 ☐
h.	Jump and land on your involved leg	4 ☐	3 ☐	2 ☐	1 ☐	0 ☐
i.	Stop and start quickly	4 ☐	3 ☐	2 ☐	1 ☐	0 ☐

FUNCTION:

10. How would you rate the function of your knee on a scale of 0 to 10 with 10 being normal, excellent function and 0 being the inability to perform any of your usual daily activities which may include sports?

FUNCTION PRIOR TO YOUR KNEE INJURY:

	0	1	2	3	4	5	6	7	8	9	10	
Couldn't perform daily activities	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	No limitation in daily activities

CURRENT FUNCTION OF YOUR KNEE:

	0	1	2	3	4	5	6	7	8	9	10	
Can't perform daily activities	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	No limitation in daily activities

Scoring Instructions for the 2000 IKDC Subjective Knee Evaluation Form

Several methods of scoring the IKDC Subjective Knee Evaluation Form were investigated. The results indicated that summing the scores for each item performed as well as more sophisticated scoring methods.

The responses to each item are scored using an ordinal method such that a score of 0 is given to responses that represent the lowest level of function or highest level of symptoms. For example, item 1, which is related to the highest level of activity without significant pain is scored by assigning a score of 0 to the response "Unable to perform any of the above activities due to knee pain" and a score of 4 to the response "Very strenuous activities like jumping or pivoting as in basketball or soccer". **For item 2, which is related to the frequency of pain over the past 4 weeks, the responses are reverse-scored such that "Constant" is assigned a score of 0 and "Never" is assigned a score of 10. Similarly, for item 3, the responses are reversed-scored such that "Worst pain imaginable" is assigned a score of 0 and "No pain" is assigned a score of 10.** Note: previous versions of the form had a minimum item score of 1 (for example, ranging from 1 to 11). In the most recent version, all items now have a minimum score of 0 (for example, 0 to 10). To score these prior versions, you would need to transform each item to the scaling for the current version.

The IKDC Subjective Knee Evaluation Form is scored by summing the scores for the individual items and then transforming the score to a scale that ranges from 0 to 100. **Note:** The response to item 10a "Function Prior to Knee Injury" is not included in the overall score. To score the current form of the IKDC, simply add the score for each item (the small number by each item checked) and divide by the maximum possible score which is 87:

$$\text{IKDC Score} = \left[\frac{\text{Sum of Items}}{\text{Maximum Possible Score}}\right] \times 100$$

Thus, for the current version, if the sum of scores for the 18 items is 45 and the patient responded to all the items, the IKDC Score would be calculated as follows:

$$\text{IKDC Score} = \left[\frac{45}{87}\right] \times 100$$

$$\text{IKDC Score} = 51.7$$

The transformed score is interpreted as a measure of function such that higher scores represent higher levels of function and lower levels of symptoms. A score of 100 is interpreted to mean no limitation with activities of daily living or sports activities and the absence of symptoms.

The IKDC Subjective Knee Form score can be calculated when there are responses to at least 90% of the items (i.e. when responses have been provided for at least 16 items). In the original scoring instructions for the IKDC Subjective Knee Form, missing values are replaced by the average score of the items that have been answered. However, this method could slightly over- or under-estimate the score depending on the maximum value of the missing item(s) (2, 5 or 11 points). Therefore, in the revised scoring procedure for the current version of a form with up to two missing values, the IKDC Subjective Knee Form Score is calculated as (sum of the completed items) / (maximum possible sum of the completed items) * 100. This method of scoring the IKDC Subjective Knee Form is more accurate than the original scoring method.

A scoring spreadsheet is also available at: www.sportsmed.org/research/index.asp This spreadsheet uses the current form scores and the revised scoring method for calculating scores with missing values.

KOOS KNEE SURVEY

Today's date: _____/_____/_____   Date of birth: _____/_____/_____

Name: _____

INSTRUCTIONS: This survey asks for your view about your knee. This information will help us keep track of how you feel about your knee and how well you are able to perform your usual activities.

Answer every question by ticking the appropriate box, only <u>one</u> box for each question. If you are unsure about how to answer a question, please give the best answer you can.

Symptoms

These questions should be answered thinking of your knee symptoms during the **last week**.

S1. Do you have swelling in your knee?

Never	Rarely	Sometimes	Often	Always
☐	☐	☐	☐	☐

S2. Do you feel grinding, hear clicking or any other type of noise when your knee moves?

Never	Rarely	Sometimes	Often	Always
☐	☐	☐	☐	☐

S3. Does your knee catch or hang up when moving?

Never	Rarely	Sometimes	Often	Always
☐	☐	☐	☐	☐

S4. Can you straighten your knee fully?

Always	Often	Sometimes	Rarely	Never
☐	☐	☐	☐	☐

S5. Can you bend your knee fully?

Always	Often	Sometimes	Rarely	Never
☐	☐	☐	☐	☐

Stiffness

The following questions concern the amount of joint stiffness you have experienced during the **last week** in your knee. Stiffness is a sensation of restriction or slowness in the ease with which you move your knee joint.

S6. How severe is your knee joint stiffness after first wakening in the morning?

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

S7. How severe is your knee stiffness after sitting, lying or resting **later in the day**?

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

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Pain

P1. How often do you experience knee pain?

Never	Monthly	Weekly	Daily	Always
☐	☐	☐	☐	☐

What amount of knee pain have you experienced the **last week** during the following activities?

P2. Twisting/pivoting on your knee

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P3. Straightening knee fully

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P4. Bending knee fully

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P5. Walking on flat surface

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P6. Going up or down stairs

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P7. At night while in bed

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P8. Sitting or lying

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P9. Standing upright

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

Function, daily living

The following questions concern your physical function. By this we mean your ability to move around and to look after yourself. For each of the following activities please indicate the degree of difficulty you have experienced in the **last week** due to your knee.

A1. Descending stairs

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A2. Ascending stairs

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

For each of the following activities please indicate the degree of difficulty you have experienced in the **last week** due to your knee.

A3. Rising from sitting

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A4. Standing

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A5. Bending to floor/pick up an object

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A6. Walking on flat surface

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A7. Getting in/out of car

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A8. Going shopping

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A9. Putting on socks/stockings

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A10. Rising from bed

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A11. Taking off socks/stockings

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A12. Lying in bed (turning over, maintaining knee position)

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A13. Getting in/out of bath

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A14. Sitting

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A15. Getting on/off toilet

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

For each of the following activities please indicate the degree of difficulty you have experienced in the **last week** due to your knee.

A16. Heavy domestic duties (moving heavy boxes, scrubbing floors, etc)
None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A17. Light domestic duties (cooking, dusting, etc)
None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

Function, sports and recreational activities
The following questions concern your physical function when being active on a higher level. The questions should be answered thinking of what degree of difficulty you have experienced during the **last week** due to your knee.

SP1. Squatting
None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

SP2. Running
None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

SP3. Jumping
None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

SP4. Twisting/pivoting on your injured knee
None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

SP5. Kneeling
None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

Quality of Life

Q1. How often are you aware of your knee problem?
Never	Monthly	Weekly	Daily	Constantly
☐	☐	☐	☐	☐

Q2. Have you modified your life style to avoid potentially damaging activities to your knee?
Not at all	Mildly	Moderately	Severely	Totally
☐	☐	☐	☐	☐

Q3. How much are you troubled with lack of confidence in your knee?
Not at all	Mildly	Moderately	Severely	Extremely
☐	☐	☐	☐	☐

Q4. In general, how much difficulty do you have with your knee?
None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

Thank you very much for completing all the questions in this questionnaire.

TEGNER ACTIVITY LEVEL SCALE

Please indicate in the spaces below the HIGHEST level of activity that you participated in BEFORE YOUR INJURY and the highest level you are able to participate in CURRENTLY.

BEFORE INJURY: Level_____   CURRENT: Level_____

Level 10	Competitive sports- soccer, football, rugby (national elite)
Level 9	Competitive sports- soccer, football, rugby (lower divisions), ice hockey, wrestling, gymnastics, basketball
Level 8	Competitive sports- racquetball or bandy, squash or badminton, track and field athletics (jumping, etc.), down-hill skiing
Level 7	Competitive sports- tennis, running, motorcars speedway, handball Recreational sports- soccer, football, rugby, bandy, ice hockey, basketball, squash, racquetball, running
Level 6	Recreational sports- tennis and badminton, handball, racquetball, down-hill skiing, jogging at least 5 times per week
Level 5	Work- heavy labor (construction, etc.) Competitive sports- cycling, cross-country skiing, Recreational sports- jogging on uneven ground at least twice weekly
Level 4	Work- moderately heavy labor (e.g. truck driving, etc.)
Level 3	Work- light labor (nursing, etc.)
Level 2	Work- light labor Walking on uneven ground possible, but impossible to back pack or hike
Level 1	Work- sedentary (secretarial, etc.)
Level 0	Sick leave or disability pension because of knee problems

Y Tegner and J Lysolm. *Rating Systems in the Evaluation of Knee Ligament Injuries.* Clinical Orthopedics and Related Research. Vol. 198: 43-49, 1985.

Subject ID: ⬚⬚⬚⬚⬚⬚   **Date:** ⬚⬚ / ⬚⬚ / ⬚⬚
                   Month   Day   Year

Time In: ⬚⬚⬚

Time Out: ⬚⬚

EATA Data Collection Form – Baseline & Biomechanics

ACL Leg: R   or   L   or   N/A   **Dominant Leg:** R   or   L

Mass: _____ kg   (w/shoes)   **Survey Forms:**   IKDC   KOOS   Tegner

Height: _____ m   **Self-selected speed (m/s):** _____

Qualisys Project: MW_Treadmill_EATA   **First speed condition:** 120% SS   or   80% SS

Trial	Speed Condition			Limb	Comments
1	SS	120%	80%	R or L	
2	SS	120%	80%	R or L	
3	SS	120%	80%	R or L	
4	SS	120%	80%	R or L	
5	SS	120%	80%	R or L	
6	SS	120%	80%	R or L	
7	SS	120%	80%	R or L	
8	SS	120%	80%	R or L	
9	SS	120%	80%	R or L	
10	SS	120%	80%	R or L	
11	SS	120%	80%	R or L	
12	SS	120%	80%	R or L	
13	SS	120%	80%	R or L	
14	SS	120%	80%	R or L	
15	SS	120%	80%	R or L	
16	SS	120%	80%	R or L	
17	SS	120%	80%	R or L	

EATA Data Collection Form – Quadriceps Strength

Limb Randomization (Circle One):   R   **or**   L

Instructions:
- Setup Cybex with **"BFB_QuadStrength.Vi"** LabVIEW Program
- Instruct subject to sit in dynamometer chair
- Examiner to secure chest, waist, and leg straps to participant
- Position chair and dynamometer arm so that dynamometer arm aligns with knee joint center
- Dynamometer arm pad should be position **two finger widths above the medial malleolus.**
- Cybex should be set to measure strength at 60 deg/sec in Con/Con mode, Isometrics @ 60 deg.
- Instruct subjects that they are to kick as hard as they can out and pull as hard as they can in; Examiners to provide vigorous encouragement
- **Practice Trials:** 1 repetition at 25%, 50%, 75%, and 100% of perceived maximal effort.

Chair/Dynamometer Positions:
Dynamometer Arm: _____
Dynamometer Rotation: _____
Chair Fore/Aft: _____
Chair Height: _____

Quadriceps and Hamstring Isokinetic Strength (60-degrees/s):

Trial #	Right Leg Extension	Right Leg Flexion	Left Leg Extension	Left Leg Flexion
1				
2				
3				
Average				

Quadriceps Isometric Strength (60-degree Knee Angle):

Rep #	Right Leg			Left Leg		
	Peak Torque	RTD 50-100ms	RTD 100-200ms	Peak Torque	RTD 100	RTD 200
1						
2						
3						
Average						

18	SS	120%	80%	R or L	
19	SS	120%	80%	R or L	
20	SS	120%	80%	R or L	
21	SS	120%	80%	R or L	
22	SS	120%	80%	R or L	
23	SS	120%	80%	R or L	
24	SS	120%	80%	R or L	
25	SS	120%	80%	R or L	
26	SS	120%	80%	R or L	
27	SS	120%	80%	R or L	
28	SS	120%	80%	R or L	
29	SS	120%	80%	R or L	
30	SS	120%	80%	R or L	
31	SS	120%	80%	R or L	
32	SS	120%	80%	R or L	
33	SS	120%	80%	R or L	
34	SS	120%	80%	R or L	
35	SS	120%	80%	R or L	
36	SS	120%	80%	R or L	
37	SS	120%	80%	R or L	
38	SS	120%	80%	R or L	
39	SS	120%	80%	R or L	
40	SS	120%	80%	R or L	

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