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ANTERIOR CRUCIATE LIGAMENT INJURY-INDUCED ALTERATIONS IN INFLAMMATION AND MUSCLE PHYSIOLOGY

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ANTERIOR CRUCIATE LIGAMENT INJURY-INDUCED ALTERATIONS IN INFLAMMATION AND MUSCLE PHYSIOLOGY

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Health Sciences
at the University of Kentucky

By

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ABSTRACT OF DISSERTATION

ANTERIOR CRUCIATE LIGMAENT INJURY-INDUCED ALTERATIONS IN INFLAMMATION AND MUSCLE PHYSIOLOGY

Long term weakness and atrophy of the quadriceps muscle are a direct result of anterior cruciate ligament (ACL) injuries and persist for up to 10 years post injury. Muscle atrophy ensues regardless of ligamentous reconstruction surgery, indicating that muscle atrophy following injury is a function of the ligament rupture and not reconstruction surgery. Elucidating the mechanisms underlying quadriceps atrophy following ACL rupture is crucial for developing interventions to restore proper quadriceps size and mitigate weakness thereby allowing for improved patient function. In addition to understanding the specific mechanisms that contribute to quadriceps atrophy following ACL rupture, the timing of atrophic events that cause the decreased function of the quadriceps muscle must be elucidated to determine the best intervention strategy. Most studies involving human subjects are conducted post ACL reconstruction with limited emphasis on the cellular changes in muscle that could be responsible for the observed atrophy and weakness. Animal models involving open-knee surgery for ACL transection have been employed to investigate cellular mechanisms in muscle, but these models are not mimicking the human ACL rupture, which is a closed-knee injury. Thus, there is a need for fundamental research of the timing and underlying mechanisms of inflammation and quadriceps atrophy following ACL injury.

The purpose of this dissertation is twofold: Firstly, to examine the relationship between serum and synovial joint inflammatory markers and proteomics in ACL deficient subjects; and to use those acquired data to identify potential cellular processes that may influence skeletal muscle and track those processes longitudinally in conjunction with changes in muscle size following a closed ACL injury in a rat model. The goals of these studies were to determine if a closed ACL rupture in the absence of surgical repair would induce persistent quadriceps atrophy, determine the relationship between ACL rupture and intra-articular and systemic inflammation, and investigate time-dependent cellular changes in the vastus lateralis muscle. Results indicate there is an increase in inflammatory cytokines (IL-1β (interleukin 1-beta) and IL-6(interleukin six)) in both the synovial joint fluid and systemic circulation following injury in humans. In

the in vivo animal model of ACL rupture quadriceps atrophy was observed one week post injury, but muscle size unexpectedly recovered as early as 2 weeks. Gene expression of MAFbx and Murf-1 (atrophy markers) was elevated as early as 48 hours after ligament rupture. No signs of muscle damage were observed at any time point. This dissertation provides the first longitudinal investigation into the changes in muscle physiology following a closed ACL-rupture, informed by a proteomic analysis of human serum and synovial fluid after an acute clinical ACL tear. The results of these studies begin to characterize a relationship between joint inflammation and muscle atrophy in the acute time period following ACL injury.

KEYWORDS: Anterior Cruciate Ligament Injury, Inflammation, Quadriceps, Atrophy

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CHAPTER 1. INTRODUCTION

Background

Anterior cruciate ligament (ACL) tears are one of the most common orthopedic injuries affecting more than 200,000 people a year, of all ages and activity levels.[1] The complete tearing of this static stabilizer in the knee causes a cascade of events that ultimately leads to long-term dysfunction of the knee joint and quadriceps muscle. The quadriceps muscle is a group of four muscles working synergistically to extend the knee and provide dynamic stabilization and shock absorption.[2] Persistent quadriceps weakness and atrophy is a hallmark of ACL injury and arguably the most detrimental result. Atrophy and weakness of this muscle group persists long after the injury and even surgical intervention causing joint instability,[1,3] altered gait[4], muscle activation failure[5], and may contribute to the development of osteoarthritis.[6,7]

In the wake of an ACL rupture there is a sustained increase in intraarticular inflammatory cytokines and cartilage breakdown markers present in the joint.[8-14] The increase in inflammation within the synovial environment is important because chronic inflammation could be a contributing factor to alterations in the systemic environment as well as joint degradation[15] and potential muscle atrophy.[16] The exact mechanism leading to quadriceps atrophy following injury is still unknown, but a link between the quadriceps and neural alterations has been well established.[17-19] There is also evidence to support a potential cellular cross-talk between the joint and the muscle[20] indicating that alterations to the quadriceps following ACL injury is multifactorial. Research efforts focused on the effects of ACL rupture on the quadriceps muscle tend to be confounded by limitations in human research and studies whose patient populations are post-surgical.

ACL rupture causes a deleterious cascade of events that includes joint inflammation, changes to the systematic environment and alterations to the quadriceps muscle. Changes to the local, systemic and muscular environment likely all contribute to the atrophy and muscle weakness seen clinically and elucidating the mechanisms responsible for atrophy and quadriceps dysfunction will aid in the development of interventions and treatment. The goal of ACL reconstruction surgery is to reinstate joint stability but even this stabilizing surgery has the potential to present an additional inflammatory burden to the joint[21] and create an environment of sustained inflammation. This dissertation will begin to dissect how ACL injury affects the synovial fluid environment, the circulating serum in human patients and the quadriceps muscle using a novel pre-clinical animal model.

Quadriceps Atrophy Regardless of Reconstruction

The current standard of care in the US is to reconstruct an ACL after injury surgically in order to restore biomechanical stability. Human research centered around the changes in muscle morphology following ACL injury is mostly cross-sectional in design and confounded by the presence of joint surgery. Non-operative treatment of ACL injury is less common, but the question becomes whether patients that remain ACL deficient also undergo weakness and atrophy like those who have surgery. Conservative treatment following ACL injury is centered around regaining quadriceps strength along with normal mobility. The quadriceps and hamstring muscle groups have to be working synergistically in order for the knee joint to regain normal biomechanical function post ACL injury and the strength in both groups should be comparable to that on the non-injured leg.

As far back as the 1960s Edstrom[22] showed muscle atrophy in myosin type I fiber atrophy following chronic ACL injury. His group followed eight cases of patients with torn ACLs and demonstrated that five out of the eight patients with a chronic ACL injury had significant statistical differences in fiber type cross sectional area between the "red" and "white" fibers. Edstrom made a case of selective atrophy of the quadriceps muscle due to disuse.[22] Tegner et al. in 1984[23] measured the isokinetic quadriceps and hamstring strength of sixteen patients with "old" anterior cruciate ligament tears after undergoing a twelve-week long thigh musculature strengthening program. Researchers found that before the training period quadriceps strength was significantly decreased compared to the non-injured limb. Over the next two time points, quadriceps strength increased but never reached the level of the non-involved limb and remained significantly different.[23] These studies indicate that patients who suffer from ACL rupture will have decreased strength long term even after rehabilitation.

Chapter two of this dissertation will discuss whether conservative treatment of ACL injury yields outcomes similar to reconstruction surgery. Patient reported function is not different between patients treated non-operatively and patients treated with reconstruction suggesting that surgery is not necessary to be functional in all cases.[24] There is also evidence to suggest that the severity of the injury may have a role in the development of quadriceps weakness but overall those with a complete tear of the ACL had poor to fair muscular strength eight years after injury.[25] In a comprehensive study done by Tsepis in 2006[26], patients with an ACL injury were broken up into three groups depending on the length of time since injury. The short time group (less than six months) and then a medium (6 to 18 months) and a long-term group (average of 56 months). Quadriceps

strength was decreased by at least 20% percent for all groups compared to healthy control subjects.[26] This shows a persistence of muscle weakness and decrease in cross sectional area[5] well after injury and without reconstruction. While some studies showed less weakness such as Patel et al., 2003[27] and Kvist et. al, 2001[28], all of the studies reported at least a 7% deficit in involved quadriceps strength. Chapter two of this dissertation is a critically appraised topic that will discuss the effect of non-operative ACL treatment on isokinetic muscle strength, function and patient reported function. Atrophy and weakness of the quadriceps muscle persists regardless of reconstruction, and patients who remain ACL deficient (ACLD) will continue to have quadriceps atrophy and joint dysfunction years following ACL injury. Research focused on the specific alterations in quadriceps function before reconstruction is lacking and there is a need for longitudinal studies to begin to fill the gaps in knowledge.

Cellular Changes in Quadriceps muscle Following ACL Injury

To start to identify the potential mechanisms of quadriceps atrophy after ACL rupture, researchers have turned to cellular measures of muscle morphology. Recent literature reveals differences in physiological cross sectional area (CSA) between injured and non-injured limbs with the largest decrease in muscle size in the vastus lateralis.[29] Furthermore, there was a specific decrease in the CSA of type-IIA muscle fibers preceding surgery. After reconstruction surgery, the frequency of type IIA muscle fibers significantly decreased in conjunction with increases in type IIA/IIX hybrid fiber types.[30] Muscle biopsies from the vastus lateralis of patients with an ACL injury reveal differences in physiological cross sectional area and pennation angle[30], both of which are important for proper function of the quadriceps group. Following ACL injury there are

also significant increases in fibroblast and collagen content which correlated with increases in extracellular matrix.[30,31] This is important because increases in extracellular matrix could potentially limit hypertrophy[32] and therefore adversely affect function and recovery from injury. Moreover, the injured limb showed decreases in satellite cell number[30,31], which could lead to a decreased capacity for muscle growth. Additionally, muscle biopsies from the injured limb had significantly higher myostatin gene expression and protein abundance.[33] Myostatin is a potent negative regulator of muscle size[34] and increases in myostatin may be an important factor in quadriceps atrophy following ACL injury. In addition, myostatin and TGF-β levels in serum were also increased following ACL reconstruction.[35] Along with TGF-β, muscle from the injured limb also has increased protein abundance of the inflammatory cytokines IL-6 and TNFα. [33] Elevations in proinflammatory cytokines in serum mirror what is occurring in the joint post injury, for example elevations in both synovial fluid pro-inflammatory cytokines like IL-6 are similar to increases in cytokine receptor pathways in human serum. Increases in inflammatory cytokines may lead to a state of sustained inflammation providing a connection between the two environments. Changes in cross sectional area, extracellular matrix, muscle growth regulators and satellite cells provide compelling evidence that ACL injury causes a myriad of detrimental changes to the quadriceps muscle. There are also increases in apoptosis and denervated muscle fibers[31] which could potentially explain some of the neurogenic response seen in patients after this injury. There is an abundance of literature to support alterations in neural feedback in the quadriceps after injury.[17-19] Increases in denervated muscle fibers could begin to explain the quadriceps inhibition and lack of motor recruitment that is associated with weakness after ACL

rupture. This evidence demonstrates changes within the muscle itself following injury that likely work in combination to create changes within the neural feedback network that contributes to weakness.

The literature presented above provides insight into understanding the complicated response of the quadriceps muscle to ACL rupture. It should be noted that the average time of the muscle biopsies in the above studies was 60 days post injury and therefore early events underlying muscle changes remain largely unknown. There is also a limitation in the number of muscle biopsies that can be collected from human subjects with the majority of studies presented here just looking at biopsies from pre- to post-reconstruction. Surgery presents a new insult to the joint and it is still unclear if that insult will further modify the quadriceps. Only having one time point pre-surgery prohibits a complete picture of longitudinal changes occurring after injury from being put together. To augment the knowledge base while there is a lack of longitudinal human studies, animal models of ACL injury allow for a more in depth analysis of the quadriceps.

Animal Models of ACL Injury

The use of animal models allows researchers to study multiple time points post injury. Additionally, with animal models comparison between injured and non-injured limbs can be easily obtained. Also, animal studies provide more feasibility in the timing of sample collection allowing for longitudinal studies targeted at determining quadriceps changes during the acute post-injury period. Studies utilizing ACL transection models examined changes in quadriceps physiology at one, two, three, seven and fifteen days post

transection.[36,37] Both studies show significant increases in two markers of muscle catabolism Murf-1 and Mafbx from days 1-3 over the control and sham groups.[36,37] Increases in these markers are important because Murf-1 and Mafbx are two ubiquitin ligases that are associated with protein breakdown and atrophy within skeletal muscle and have been shown to be upregulated in most atrophy models. [38,39] Additionally, muscle mass loss is preceded by the upregulation of Murf-1 and Mafbx.[38,39] Similar to the human studies outlined above[33,35], significant increases in myostatin over the early post transection period that persisted out to seven days post-surgery coincide with decreases in muscle wet weight and cross sectional area.[36,37,40] Furthermore, decreases in muscle fiber CSA and significant increases in Murf-1 and Mafbx mRNA are found 60 days post ACL transection, indicating that increases in muscle atrophic signaling are present after the acute recovery period.[41]

While increases in synovial inflammation indicate that an animal model of ACL transection will undergo a similar inflammatory response to that of humans within the knee joint.[41] One important consideration when looking at animal models of ACL transection as an injury is that surgically transecting the ACL may not be as clinically relevant since surgical transection creates inflammatory and atrophic processes that mimic ACL reconstruction surgery.[36,42] The additional surgery in order to achieve transection of the ACL in traditional animal models has the potential to create an inflated inflammatory response which makes it more difficult to discern which alterations in quadriceps muscle are due to the ruptured ligament as opposed to a state of increased inflammation after a surgery. This distinction may be important as there is evidence to support that any intraarticular inflammation may play a role in muscle atrophy by

inducing Murf-1 by upregulations in NF-κβ and TNF signaling pathways.[16,43] Furthermore, it has been shown that pro-inflammatory cytokines like IL-6 have the ability to induce muscle atrophy by decreasing phosphorylation of ribosomal S6 kinase.[44] It is highly likely that inflammation plays a large role in the induction of atrophy post ACL rupture and therefore ACL transection models requiring open surgery may not be best to study this injury since an increase in inflammation will confound the effects to the quadriceps muscle.

Goal of Dissertation

There is a fundamental lack of knowledge about the mechanisms underlying quadriceps atrophy following ACL injury. There is evidence for cellular changes to the quadriceps muscle, but in humans the data are primarily cross sectional and lacks consistent study time points. Moreover, human studies have mainly focused on quadriceps atrophy following reconstruction and not just injury. However, atrophy is present regardless of ACL reconstruction indicating that the initial ligament rupture is a contributing factor. There is a need for a clinically relevant non-transection model of ACL injury to longitudinally track alterations in the quadriceps muscle.

In this dissertation we proposed to examine the connection between synovial joint inflammation and quadriceps atrophy following ACL injury in both human and animal models. Quadriceps deficits following injury are present with or without reconstruction, indicating that quadriceps atrophy is present even in ACL deficient patients. Next, in chapter three, we examine the inflammatory response in human synovial fluid following ACL injury and see that injury causes a sustained increase in proinflammatory cytokines

IL-1β and IL-6. Based on the inflammatory response in the synovial joint, human serum was profiled to determine if increases in circulating markers of inflammation was similar to synovial fluid and if these proteins have the potential to influence the catabolic balance of skeletal muscle. As such, chapter four chapter focuses on human serum proteomics following ACL injury. The results of that study indicate systemic increases in not only inflammation but also muscle specific markers like myoglobin, which traditionally used as a "stress" marker for muscle and while this specific marker was not pursued farther, it raised questions about potential muscle damage following ACL injury. Our hypothesis is that the vastus lateralis is subjected to damage during ACL tear leading to atrophy and dysfunction. To test this hypothesis, a novel in vivo animal model of ACL tear was used. The model consists of a device that will mechanically overload the tibia in relation to the femur, tearing the ACL. By tearing the ACL in a closed system where there is no increase in inflammation from a surgery, makes this clinically relevant model of injury. Our team now has the ability to study the quadriceps muscle acutely after injury allowing us to answer questions about the quadricep muscles response to ACL rupture alone at various time points after injury. Chapter five describe results from the animal model focusing on damage and atrophy markers acutely after ACL rupture.

CHAPTER 2. THE EFFECTIVENESS OF NON-OPERATIVE TREATMENT FOR ANTERIOR CRUCIATE LIGAMENT RUPTURE ON PATIENT REPORTED OUTCOMES AND MUSCULAR STRENGTH: A CRITICALLY APPRAISED TOPIC

Abstract

Clinical Scenario: Anterior Cruciate Ligament (ACL) ruptures are one of the most common injuries in young athletic populations. The leading treatment for these injuries is anterior cruciate ligament reconstruction (ACL-r), however, non-operative treatments are also utilized. Following ACL-r, patients experience prolonged muscle weakness and atrophy of the quadriceps muscle group, regardless of rehabilitation. Non-operative treatment plans following ACL injury exist, but their outcomes are less familiar, in spite of providing insight as a non-surgical 'control' for post-surgical rehabilitation outcomes. Therefore, the purpose of this critically appraised topic (CAT) was to evaluate quadriceps strength and function following non-operative ACL rehabilitation using objective and subjective measures including: Isokinetic dynamometry; the Single Leg Hop Test; and the International Knee Documentation Committee Subjective Knee Form (IKDC). *Focused Clinical Question*: In patients who have sustained an anterior cruciate ligament rupture, what are the effects of non-operative treatment on peak isokinetic knee extensor torque, the Single Leg Hop Tests and the IKDC? *Key Findings*: Patients who underwent non-surgical ACL treatment produced limb symmetry index (LSI), the side-to-side torque difference expressed as a percentage, values at or above 90% for all four Single Leg Hop Tests and strength tests similarly to ACL-r patients. All studies showed individuals had higher IKDC scores at baseline collection when compared to patients who underwent ACL-r but showed lower IKDC scores at long term follow up compared to ACL-r patients. *Clinical Bottom Line*: Non-operative treatments of ACL injuries yield similar

long-term results in quadriceps strength as ACL-r. Due to the quality of evidence and the absence of randomized controlled trials on this topic, these outcomes should be considered with caution. *Strength of Recommendation*: The Oxford Centre for Evidence-Based Medicine taxonomy recommends a grade of B for level 2 evidence with consistent findings.

Keywords: ACL, treatment, muscle strength, function

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Clinical Scenario

Anterior cruciate ligament (ACL) injuries, are among the most common traumatic athletic injuries, affecting upwards of 200,000 individuals per year in the United States (US).[1] For those US patients who wish to continue participation in competitive athletics, ligament reconstruction has become the preferred method of treatment.[45] In the US, the rate of ACL reconstruction continues to rise annually, reaching 52 reconstructions per 100,000 of the population in 2015.[46] This rate is second to Australia, who continues to lead the world with 77.4 ACL reconstructions per 100,000 citizens, with the greatest increase in ACL reconstructions among those 4-15 years of age.[47]

The primary purpose for ACL reconstruction (ACL-r) is the restoration of joint congruity concomitant with increased knee stability.[48] However, despite the advancements and improvements in surgical techniques, instrumentation, and post-surgical care and rehabilitation, individuals who undergo ACL–r inevitably suffer from persistent quadriceps atrophy and weakness long term.[49] These unresolved strength

deficits decrease athletic performance, increase the risk of re-injury,[50] and likely contribute to the progression of cartilage damage within the joint.[51] Therefore, the dogma that ACL-r is the most efficacious treatment following ACL injury should be taken with caution. In a twenty-one-year population study, Sanders et al., found that out of almost two thousand patients, 75% of them underwent ACL-**r** and 98% of those were under the age of 18.[24] Although the rates of non-operative treatment of ACL injury continue to lag behind the rate of surgical intervention in the US, there is growing support within the international community for conservative treatment. Therefore, the purpose of this critically appraised topic is to review the best, and most current literature that measured the effects of conservative, non-operative ACL treatment on quadriceps strength and patient perceived function using Isokinetic dynamometry; the Single Leg Hop Test; or the International Knee Documentation Committee Subjective Knee Form (IKDC).

Focused Clinical Question

In patients who have sustained an anterior cruciate ligament rupture, what are the effects of non-operative treatment on isokinetic muscle strength, the Single Leg Hop Tests and the IKDC-2000?

Search Strategy

In November of 2018, we performed a comprehensive computerized search using the following terms (Figure 2.1), using the PICO strategy. [52]

- Patient/Client group: *anterior cruciate ligament rupture*
- Intervention: *non-operative treatment protocol*

- Comparison: *ligamentous reconstruction OR no*
- Outcome: *patient reported outcomes AND muscular strength*

2.1.1 Sources of Evidence Search

- **EBSCOhost**
  - CINAHL
  - SPORTDiscus
  - Medline
  - Additional resources obtained via review of reference list and hand search

Inclusion and Exclusion Criteria

The criteria for inclusion were as follows:

- Studies that investigated the effectiveness of non-operative treatments
- Studies that investigated ACL ruptures
- Studies that included outcome measures of patient reported outcomes or muscular strength
- Limited to studies published within the past 10 years (2008-2018)
- Limited to English
- Level 2 evidence or higher

The criteria for exclusion were as follows:

- Studies that investigated non-ACL ruptures (i.e. ACL partial thickness tear)
- Studies that investigated other subsequent pathologies because of ACL injury

- Studies that did not investigate the effects of non-operative treatment on ACL injury
- Studies that were not in English
- Level 3 evidence or lower
- Systematic reviews and meta-analysis

2.1.2 Evidence of Quality Assessment

The Strengthening of Reporting of Observational studies in Epidemiology (STROBE) Statement was used to determine the validity of the included studies. Two authors (EH, CP) independently scored and reviewed each of the articles, after review both authors (EH, CP) reached an agreement about study quality and inclusion.

Summary of Search and Key Findings

- The literature search returned 43 studies (Figure 2.1) related to the clinical question. After review 39 studies were excluded because they did not meet the inclusion criteria for this CAT. Four prospective cohort studies[48,53-55] met inclusion criteria and are described in Table 1.
- Each included study evaluated the effects of non-operative treatment algorithms for ACL injury or compared conservative treatment and ACL-r.
- Collectively, the four studies (Table 2.1) reported significant improvements in both patient reported outcomes and muscular strength when comparing long term follow up to the time of injury. In addition, in the studies that compared the effects of non-

operative treatment with ACL-r, outcome measures were similar between the two groups.

Results of Evidence Quality Assessment

Four studies were identified as the best evidence and selected for inclusion in this critically appraised topic (CAT). The four studies were selected because they were level 2 evidence and had STROBE scores of at least 17/22. Three studies [48,54,55] examined the effects of non-operative treatment on isokinetic strength of the quadriceps and hamstrings. In addition, studies conducted by Moksnes et al., [48,53,54] examined the effects of non-operative treatment on strength using the Single Leg Hop Test and Moksnes et al. 2009, was the only study to compare the non-operative ACL group directly to the ACL-r group**.** All four studies examined the effects of non-operative ACL treatment on the IKDC 2000. [48,53-55] Moksnes et al., 2008 received a validity score of 18/22 for failure to include the study design in the abstract, address potential sources of bias and discuss the external validity of the study. Moksnes et al., 2013 did not discuss potential confounders of each variable or the external validity of the study.

Clinical Bottom Line

There is strong evidence to support that conservative treatment of ACL injuries obtains similar outcomes as ACL ligamentous reconstruction at long-term follow up periods. All studies included in this critically appraised topic,[48,53-55] generated similar results: at follow up patients who underwent non-surgical treatment of ACL injury produced LSI values at or above 90% for all four Single Leg Hop Test, as well as, knee extensor and flexor isokinetic muscular strength. In addition, all studies showed

individuals had higher IKDC-2000 scores (higher scores indicate better knee function) at baseline collection when compared to patients who underwent ACL-r but showed lower IKDC-2000 scores at long term follow up compared to ACL-r patients. There is grade B evidence that non-operative treatment yields satisfactory results in the long term. The Oxford Centre for Evidence-Based Medicine taxonomy recommends a grade of B for level 2 evidence with consistent findings.[56]

Implications for Practice, Education and Future Research

The prevalence of ACL injuries in athletes is increasingly escalating. While ACL-r is the current favored treatment, it is important that all treatment options are carefully examined in order to provide patients with the most efficient and effective care. Therefore, this critically appraised topic was completed to evaluate the best literature regarding the use of non-operative treatment regimens for ACL injuries. Four cohort studies were included in the investigation. From these four studies, the main findings were concluded that non-surgical treatment of ACL injuries could yield satisfactory results when outcome measures include the IKDC-2000 at baseline, Single Leg Hop Test, and isokinetic muscle strength.

The IKDC is a knee specific, patient reported assessment tool used to evaluate symptoms, function during daily activities and levels of symptom free sports-activity.[57] It has been determined to be a reliable and valid instrument for the outcome measures mentioned above and was therefore seen as an appropriate tool to be used in this critically appraised topic.[58,59] However, it should be noted that in a study conducted by Kessler et al.,[60] the IKDC scores were determined based upon tibio-femoral anterior-posterior

translation. Because patients who undergo ligamentous reconstruction have inevitable restoration of ligamentous stability, the comparison of anterior-posterior translation between groups of non-operative and ACL-r groups is not informative. Due to the use of this criteria as a scoring variable, future studies should take caution when using the IKDC as a tool of comparison between conservatively treated and ACL-r groups. It is important to note that while patient reported outcomes are reliable and valid tools, they are subjective to normal patient function and how the patient feels during a given time-frame. Several studies[48,55] showed that patients who opted to go through non-operative treatment presented with better IKDC-2000 scores at baseline. However, the patient population of these non-operative groups was significantly older than the patients who decided to go through with ACL-r and had an altogether lower activity level. This suggests that activities of daily living and pre-injury activity level are important factors when it comes to deciding between ACL-r and conservative treatment options.

Unfortunately, persistent quadriceps weakness has become a comorbidity to ACL-r and has spurred many novel treatments, rehabilitation programs and medical technologies in attempts to address this issue. However, strength deficits of 20% lasting for over two years have still been documented following ACL-r[61-63]. In three of the four studies included in this critically appraised topic, isovelocity torque output was used as a measure of quadriceps and hamstring strength in subjects treated conservatively following ACL injury[53-55] All three studies used the following standard procedure: 10-minute warm-up on a stationary bike followed by four practice trials performed with submaximal effort at 60°/sec. Finally, five test repetitions were performed with maximal effort at the same angular velocity of 60°/sec, starting with the uninvolved limb. Strength

deficits between extremities were reported as a limb symmetry index (LSI), or simply the side-to-side torque difference expressed as a percentage (peak torque of involved limb)/ (peak torque of uninvolved limb) x 100. Although LSI values <90% are considered abnormal,[64] Moksnes et al. 2007, and Moksnes et al. 2013 all reported quadriceps and hamstring LSI values at or above 90% at baseline and follow up. Moreover, Grindem et al. 2014 further reported that only nine out of 43 non-surgically treated patients exhibited quadriceps and hamstring LSI values of <90% at follow up. These findings demonstrate that non-operative treatments of ACL injuries have the possibility to produce desirable muscle strength.

The Single Leg Hop Tests have been shown to be reliable and valid tests used to measure neuromuscular control and strength in the limb.[65-67] The test only requires a standard tape measure making it accessible to clinicians in a variety of settings, and deficits between extremities can easily be reported as LSI values. Moksnes et al. 2013 reported LSI values at or above 90% for all Single Leg Hop Tests at baseline and at all follow up periods (1 year and 2 years). Additionally, Moksnes et al. 2009 and Grindem et al. 2014 reported LSI values above 90% for all Single Leg Hop Tests at follow up, demonstrating that non-surgical treatments of ACL ruptures can improve neuromuscular control, strength and confidence as measured by the Single Leg Hop Test.

Interestingly, all of the studies included in this CAT report baseline LSI values at or above 90%. This could be due to baseline outcomes being measured at least 6 months after injury. Outcome measures examined by Moksnes et al. 2008 did not get evaluated until at least two years after ACL injury. Additionally, it was routine for patients that were included in these studies to undergo pre-operative rehabilitation sessions to resolve

initial impairments of ACL injury. Recently, it was shown that patients who underwent ACL-r were typically returned to contact sports within six-months post-surgery.[64,68] Gridnem et al., reported that athletes who were treated non-operatively returned primarily to non-pivoting sports. Whether functional and strength differences exist between conservative and surgical treatment at the time when most highly active individuals are returned to sport is unknown and should be the focus of future studies.

The findings of this CAT imply that non-operative treatments of ACL injuries are adequate options in restoration of patient function, as well as patient strength at long term follow ups. However, patient goals, normal activities of daily living and sport activity level should all be taken into consideration when choosing the best treatment plan for patients. Furthermore, the absence of randomized control trials on this topic hinder the strength of these recommendations. While further investigation regarding this topic is warranted, patients should be thoroughly informed of all treatment options available. Evidence suggests that conservative treatment has the ability to generate positive outcomes in the long term and should be considered for ACL-deficient individuals.

Table 2.1 Characteristics of Included Studies

	Study 1 Moksnes et al. 2008	**Study 2** Moksnes et al. 2009	**Study 3** Moksnes et al. 2013	**Study 4** Grindem et al. 2014
Study/Design	Prospective cohort study	Prospective cohort study	Prospective cohort study	Prospective cohort study
Participants	26 patients, 11 girls and 15 boys were included throughout the entirety of the study. 20 patients were treated non-operatively while 6 patients were treated with ACL-r.	At baseline, 125 patients, 56 females and 69 males (age 27.2 ± 8.6). At follow up 102 patients remained. 52 patients in the non-operative group and 50 patients in the ACL-reconstruction (ACL-r) group.	46 patients, 16 females and 30 males (age at baseline 11.8 ± 1.3). 34 patients remained in the non-operative treatment group and 10 patients opted to undergo ACL-r.	143 patients, 80 females and 63 males were included in the study. 43 patients chose to be treated non-operatively and 100 patients were treated with ACL-r.
Inclusion	ACL injury before the age of 13, two years from injury to or reconstruction to follow up period, ACL rupture confirmed by magnetic resonance imagining,	Level 1 or level 2 activities, unilateral ACL rupture.	Traumatic complete intrasubstance ACL injury sustained at the age of 12 or younger	ACL injury within past 3 months, MRI diagnosis, 3mm difference between knees using KT-1000, 13-60 years old, participate in level 1 or level 2 activities at

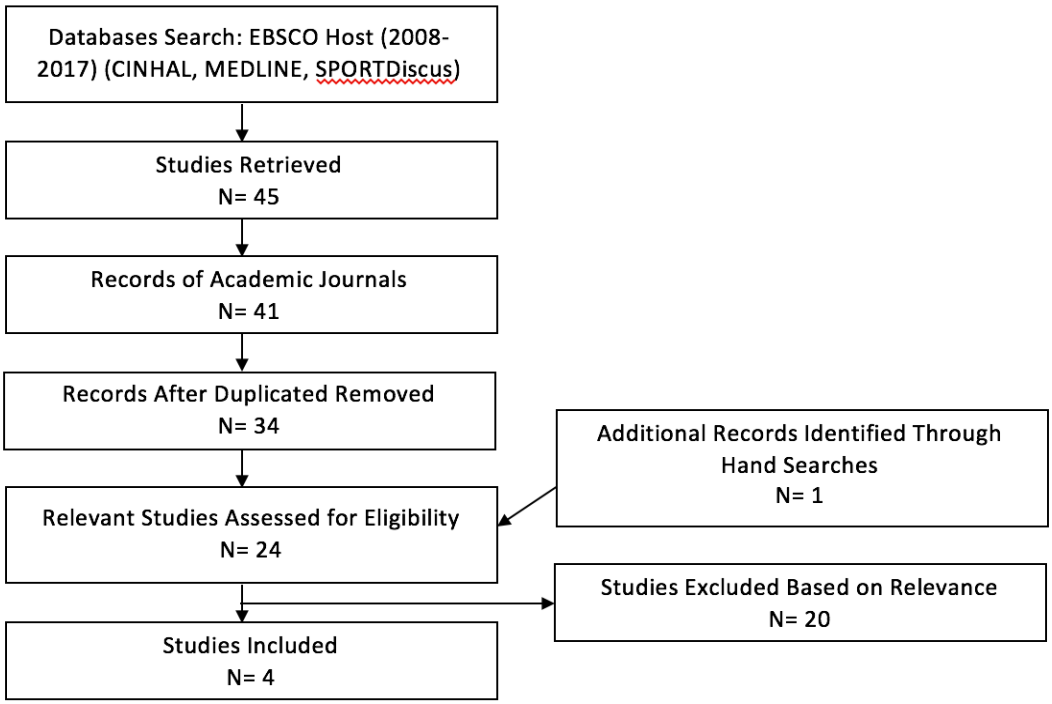
	clinical examination by one experienced orthopedic surgeon and positive Lachman's test.			least 2x a week.
Exclusion	ACL avulsion injury, posterior cruciate ligament injury or intraarticular fractures.	Posterior cruciate ligament injury, intraarticular fractures, symptomatic meniscal injury, cartilage injury affecting the subchondral bone plate, or any other injury to the leg.	Tibial or femoral ACL avulsion fractures	Current or pervious injury to contralateral leg if MRI showed another grade 3 ligament injury, fracture or full-thickness articular cartilage damage or patients with symptomatic meniscal injury
Follow up Periods	**Follow Up:** taken at a minimum of 2-years post ACL injury.	**Baseline:** Taken after 3 months of rehabilitation program. Baseline testing within first 6-months post index injury **1 Year Follow Up:** One year after baseline measurement	**Baseline:** Taken after patient completed phase two of the rehabilitation program previously published by the investigators and could perform single	**Baseline:** Taken at a mean of 2-months post injury. Before baseline, completed rehabilitation to resolve any initial impairments from the injury.

		OR one year after ACL-r.	legged hops without pain. Mean time from injury to baseline testing was 11.7 ± 11.5 months. **1 Year Follow Up:** One year post baseline **2 Year Following up:** Two-years post baseline	**Six-week test:** taken after participation in the 5-week rehabilitation protocol described by Eitzen et al. **2- year follow up:** taken 2 years after 6-week test or 2 years after ACL-r.
Outcome Measure(s)	Single legged hop tests (single hop, triple hop, triple crossover hop, 6-m timed hop), IKDC 2000, isokinetic muscle strength of knee	Single legged hop tests (single hop, triple hop, triple crossover hop, 6-m timed hop), IKDC 2000	IKDC 2000 (with the help of a parent), isokinetic muscle strength of the knee, the four Single legged hop tests.	Isokinetic muscle strength of the knee, IKDC-2000
Main Findings	IKDC mean score of 85 (71-95). All Single legged hop tests above 90% LSI. Both quadriceps and hamstring strength were above 90% LSI.	**At Baseline:** subjects who were older, performed better on the triple crossover and 6-m time hop, and had better IKDC 2000 scores opted to go through with non-operative treatment. **1 Year Follow up:** Non-	77% of children remained ACL deficient throughout 2 years. 91% of ACL deficient children continued to participate in pivoting sports. IKDC 2000 had a mean score of 82.7 at baseline and increased to an	Isokinetic muscle strength of both the hamstring and quadriceps muscle groups were at or above an LSI value of 90%. There was no significant group by time change throughout the course of the

		operative group preformed significantly better on the single and triple hop tests. ACL-r group had significantly higher IKDC 2000	82.9 at 2 year follow up. Did not show a significant increase in value over the course of the study. All single legged hop test above 90% LSI at baseline. There was a significant change in Single and 6 m timed. At baseline both quadriceps and hamstring LSI strength measurements were above 90%. No significant change in either over 2 years	study. IKDC scores increased throughout the 2-year study, again however, there was not a significant group by time relationship.
Level of Evidence	2b According to the Oxford Centre for Evidence-Based Medicine taxonomy.	2b According to the Oxford Centre for Evidence-Based Medicine taxonomy.	2b According to the Oxford Centre for Evidence-Based Medicine taxonomy.	2b According to the Oxford Centre for Evidence-Based Medicine taxonomy.
Validity Score	18/22	17/22	17/22	21/22

Conclusion	At two years after ACL injury, ACL deficient patients had adequate knee function.	A majority of the individuals who were treated with a conservative treatment algorithm were able to return to pre-injury activity level. In addition, when compared to individuals who underwent ACL-r, non-operative subjects had similar performance-based outcome measures.	Majority of children remained ACL deficient and had adequate knee function.	At 2-years post-injury, the non-surgically treated group had LSI values >90 for both knee extension and flexion. The IKDC-2000 scores improved over 2 years.

Figure 2.1 Summary of Search History and Included Studies



CHAPTER 3. ANTERIOR CRUCIATE LIGAMENT RECONSTRUCTION REINITIATES AN INFLAMMATORY AND CHONDRODEGENERATIVE PROCESS IN THE KNEE JOINT

Abstract

Anterior cruciate ligament (ACL) injury leads to a sustained increase in synovial fluid concentrations of inflammatory cytokines and biomarkers of cartilage breakdown. While this has been documented post-injury, it remains unclear whether ACL reconstruction surgery contributes to the inflammatory process and/or cartilage breakdown. This study is a secondary analysis of 14 patients (9 males/5 females, mean age=19, mean BMI=28) enrolled in an IRB-approved randomized clinical trial. Arthrocentesis was performed at initial presentation (mean=6 days post-injury), immediately prior to surgery (mean=23 days post-injury), one-week post-surgery, and one-month post-surgery. ELISA kits were used to determine concentrations of C-telopeptide fragments of type II collagen (CTXII), IL-6 and IL-1β in the synovial fluid. The log transformed IL-1β was not normally distributed; therefore, changes between time points were evaluated using a non-parametric Kruskal-Wallis one-way ANOVA. IL-1β concentrations significantly increased from the day of surgery to the first postoperative time point ($p \leq .001$) and significantly decreased at the 4-week postoperative visit ($p=.03$). IL-1β concentrations at the 4-week postoperative visit remained significantly greater than both preoperative time points ($p > .05$). IL-6 concentrations at 1 week post-surgery were significantly higher than at initial presentation ($p=0.013$), the day of surgery ($p<0.001$), and 4 weeks after surgery ($p=0.002$). CTX-II concentrations did not differ between the first three time points ($p>.99$) but significantly increased at 4 weeks post-surgery ($p<.01$). ACL reconstruction appears to reinitiate an inflammatory response followed by an increase in markers for

cartilage degradation. ACL reconstruction appears to initiate a second "inflammatory hit" resulting in increased chondral breakdown suggesting that post-operative chondroprotection may be needed.

Introduction

Anterior cruciate ligament (ACL) injury is one of the most prevalent musculoskeletal injuries affecting young athletic populations with an incidence of 200,000 persons per year.[69] Post traumatic osteoarthritis (PTOA) is an unfortunate consequence of ACL injury, with 50%-60% of patients exhibiting detectable radiographic changes consistent with osteoarthritis (OA) as early as 5 years and clinical symptoms of OA within ten years following the injury.[70,71] Previous[72,73] studies have indicated that ACL rupture begins a deleterious cascade of events that include an increase in inflammatory cytokines and collagen breakdown markers within the joint following injury.[9,11-14,74-76]

In the United States of America, the overwhelming majority of patients with ACL injuries undergo ACL reconstruction. The primary goal of ACL reconstruction is to reestablish joint stability to allow patients to return to their previous level of function.[77] Furthermore, reestablishing tibio-femoral kinematics with surgery has the potential to diminish subsequent cartilage damage;[78] however, patients often exhibit signs and symptoms of PTOA within 10 years of injury regardless of whether or not they undergo reconstruction.[79] Recent data from a large randomized clinical trial investigating surgical versus non-surgical or delayed surgical treatment of ACL injured patients has raised the concern that ACL reconstruction leads to an increase in inflammatory makers compared

to patients that did not undergo surgery. This second "inflammatory hit" has not been confirmed to date.[21]

The purpose of this study was to assess inflammatory and cartilage biomarkers before and after ACL reconstruction to determine if surgery results in a secondary inflammatory response. We hypothesized that markers of inflammation (interleukin-1β, (IL-1β), interleukin-6 (IL-6)) would be significantly increased following ACL reconstruction surgery.

Methods

3.1.1 Patients

Patients with primary ACL injury consented to enrollment in an IRB-approved randomized clinical trial assessing the use of hyaluronate injection versus placebo one week post reconstruction (clinicaltrials.gov: NCT[redacted]). There were no postoperative differences between the placebo and hyaluronate groups in terms of cartilage or inflammatory biomarkers, allowing groups to be collapsed for the current analysis. Patients were enrolled within the first 10 days following ACL injury. To be included in this study, patients had to have an isolated ACL tear with no concurrent PCL injury and could not have a grade 3 or higher MCL or LCL injury. Patients were between the ages of 14 and 32 and were skeletally mature with closed knee growth plates verified via radiograph. They had to have no history of previous surgery on the ipsilateral or contralateral knee and their ACL injury had to occur during sports activity. Exclusion criteria included the ACL injury occurring more than 10 days prior to enrollment, previous ipsilateral or contralateral knee surgery, intra-articular cortisone injection into

either knee within 3 months of injury, and a history of any inflammatory disease (Figure 3.1).

3.1.2 Study Design

The current study is a secondary analysis of the previously mentioned randomized trial. Arthrocentesis was performed on the day of initial presentation (mean = 6 days post injury), and all patients received a triamcinolone acetonide (Kenalog 40mg) injection at the time of initial presentation to dampen the inflammatory response.[12] On the day of ACL reconstruction all patients received a knee aspiration (mean = 23 days post injury), before the surgical procedures began. One week post-operatively patients received a knee aspiration, were randomized and received an injection of either hyaluronate (Gel-One®, Zimmer Biomet, Warsaw, IN) or saline. Four weeks post reconstruction all patients received a final knee aspiration. Synovial fluid was aspirated and immediately centrifuged at 3,500 rpm for 10 minutes. The supernatant was collected, aliquoted and stored at -80°C for further analysis.

3.1.3 Biomarker Assays

Synovial fluid biomarkers were assessed using commercially available enzyme linked immunosorbent assays (ELISA). Assays were run in duplicate, any samples outside of the limits of detection or quantification were rerun and intra-assay coefficients of variance were less than 9.5 for all plates. Biomarkers analyzed to assess inflammation included interleukin one beta (IL-1β) and IL-6 (Meso Scale Discovery). The biomarkers utilized in the current study follow the recommendations made by the OARSI, Arthritis Research UK Osteoarthritis, and Crystal Disease Clinical Study Group Expert Working Group.[80-82]

3.1.4 Statistical Analysis

Statistical analysis was completed using Sigmaplot v14 software. Data that were not normally distributed were log transformed.[83] For data points that were below the lowest limit of detection, the manufacturer-provided LLOD value was halved and used for computation.[84] A one-way repeated measures ANOVA was performed to determine differences between time points. The log transformed IL-1β was not normally distributed; therefore, changes between time points were determined using a non-parametric Kruskal-Wallis one-way ANOVA. A Holm-Sidak test was utilized for paired comparisons. Statistical significance was set as $P < .05$.

Results

Fourteen patients (9 males and 5 females, mean age= 19 ± 2.6, mean BMI= 28 ± 5.0) were included in these analyses. Twelve of the 14 (86%) patients presented with meniscus pathology that was addressed in surgery (Table 3.1).

IL-1β concentrations one week post-surgery (from 0.04 ± 0.05 to 0.99 ± 1.22 pg/mL) were significantly greater than initial presentation 6 days post-injury ($p=0.015$) and were also significantly greater than the day of surgery ($p<0.001$). By 4 weeks post-reconstruction IL-1β concentrations decreased but did not return back to day of surgery levels ($p=0.001$). There was a significant decrease in IL-6 from the time of initial presentation to surgery ($p<0.001$). IL-6 concentrations at 1 week post-surgery were significantly greater than initial presentation (2.06 ± 5.10 to 1427.68 ± 920.62 pg/mL, $p=0.013$), the day of surgery ($p<0.001$), and 4 weeks after surgery ($p=0.002$). Four weeks

post operatively, IL-6 concentrations remained significantly higher than the day of surgery ($p<0.001$, Figure 3.2).

Discussion

The results of this study support the theory that ACL reconstruction represents a "second hit" to the injured knee joint. There is a well-documented elevation in inflammatory and cartilage breakdown markers in synovial fluid following ACL injury.[9,11,13,74,85] Despite preoperative administration of intraarticular Triamcinolone acetonide, pro-inflammatory cytokines concentrations were significantly increased in the synovial fluid following ACL reconstruction. Furthermore, cytokine concentrations one week after surgery were greater than what was observed approximately one week after the initial insult of injury.[12] Our findings concur with previous findings of an increased inflammatory burden following ACL reconstruction.[86]

The findings from this study establish the early time frame during which the inflammatory process occurs. Joint inflammation after ACL reconstruction increases immediately after surgery, and while pro-inflammatory cytokine concentrations begin to reduce 4 weeks after surgery, they do not return to normal preoperative levels. Larsson et al. found similar results, showing an increase in synovial fluid pro-inflammatory cytokines up to 5 years post reconstruction, when compared to patients who had delayed reconstruction.[87] Consequently, this suggests that the elevations in IL-1β and IL-6 seen in some of our patients may not return to normal for 5 years or longer. Interestingly, the increase in chondral breakdown markers does not increase until 4 weeks or longer after

the ACL reconstruction.[12,88] This may suggest a time-related response of chondral breakdown to the second "inflammatory hit".

A state of increased and/or prolonged inflammation could contribute to chondrodegenerative and bony changes observed in the first two years ACL reconstruction thereby promoting the progression of PTOA. Chronic inflammatory synovitis[89,90] and several pro-inflammatory cytokines, most notably IL-1 and TNFα[91,92], have been closely linked to the progression of idiopathic OA. Furthermore, data from animal studies emphasize the role of IL-1 and TNFα in the onset and progression of OA,[93] similarly pro-inflammatory stimulation of meniscus cells increases cytokine and matrix metalloproteinase (MMP) activity.[94]

ACL reconstruction is a clinically successful procedure with patients reporting improved outcomes 10 years following surgery.[95] While ACL reconstruction successfully restores joint stability, the biologic response to injury and subsequent surgery are not sufficiently addressed by current approaches. Chronic posttraumatic inflammation and seemingly related progressive chondral degradation should likely be addressed. Hence, the treatment paradigm may need to be shifted to include treatments to address inflammation outside of the immediate healing response window as described by Anderson et al.[96]

There is evidence that biologic agents targeting the inflammatory process can reduce inflammation and can potentially mitigate the early process of chondrodegradation. For example, IL-1ra can reduce chondral lesion size and improve outcomes in animal models and in an idiopathic OA population.[97,98, 99] In a small

randomized clinical trial IL-1ra has been shown to redcue pain and effusions after ACL reconstruction.[100] Our group has reported that intraarticular administration of 40 mg of triamcinolone acetonide successfully alters the progressive increase in CTXII as marker of cartilage degradation 4 weeks after ACL injury.[12] In addition biologics such as platelet rich plasma (PRP) and even more so, bone marrow aspirate concentrate (BMAC) have been shown to contain an abundance of IL-ra and other anti-inflammatory cytokines and could potentially be treatment options that need to be considered.[101,102] Other potential interventions that aim at reducing MMP and cytokine activity after acute knee injury may alter the progression of PTOA,[103] suggesting that perioperative anti-inflammatory treatment may improve joint health following surgery.

Other modifiable factors to potentially mitigate the progression of PTOA could include the timing of surgery and/or surgical technique. Traditionally, early reconstruction was contraindicated due to the risk for the development of arthrofibrosis.[104] However, more recent literature suggests that earlier timing of ACL reconstruction with early aggressive rehabilitation is not detrimental to the joint, and outcomes following early surgery are consistent with those undergoing more traditional surgical timing.[21,105] Eriksson et. al[21], suggested that early surgery may combine the traumas of the initial injury and surgery. This would avoid a "second hit" to the joint[21] potentially avoiding the re-initiation of the cartilage breakdown process. Along with the timing of surgery, additional studies are necessary to determine if less invasive surgical approaches like bridge-enhanced ACL repair[106] or "biologic ACL repair" lessens the magnitude of the "second hit" when compared to conventional ACL reconstruction.

This pilot study is not without limitations. As this was a pilot study, the results should be used for hypothesis generation and these preliminary results do not meet the rigor necessary to modify the standard of care. However, the results of this study inform us that fully-powered RCTs examining pre- and/or postoperative anti-inflammtory or chondroprotective interventions are needed. As such, additional studies are necessary to verify our findings and to develop evidence-based approaches to potentially offset the postoperative inflammatory cascade and cartilage degradation. Second, the purpose of these analyses was to assess inflammatory and cartilage biomarkers during the first postoperative month, long term follow-up will be necessary to determine if the elevated levels of proinflammatory and chondrodegenerative biomarkers persist after ACL reconstruction and contribute to the progression of PTOA. This will be done in the future as we are following this cohort of patients prospectively. Third, this well controlled pilot study included relatively little variation in terms of the timing of surgery (average 23 days post injury). Additional studies are necessary to assess whether the timing of ACL reconstruction – either acute or delayed – may dampen the postoperative inflammation and cartilage degradation.

Conclusion

ACL injury leads to an initial inflammatory response that begins to subside over the first several weeks after injury but is associated with chondral degradation. ACL reconstruction within 3-4 weeks after ACL injury reinitiates both the inflammatory response and subsequent cartilage degradation. Even with an anti-inflammatory treatment administered post injury, this "second hit" to the joint was associated with both a larger and more prolonged inflammatory response than the initial injury itself. This insight

suggests that an early post-injury and/or postoperative intervention addressing prolonged inflammation and associated chondral degradation may be needed in order to potentially alter the progression of PTOA after ACL injury.

These results represent a critical step in understanding the complex interplay between inflammation and cartilage breakdown following ACL reconstruction. We have shown the progression of inflammatory biomarkers, both in response to the initial ACL injury and after reconstruction. This provides insights into the timing of when anti-inflammatory interventions could be most meaningfully applied. Further research is necessary to explore treatment options that are both effective in reducing the inflammatory response and cartilage breakdown to promote long-term joint health.

Table 3.1 Meniscus pathology addressed at the time of ACL reconstruction

	Medial	**Lateral**
No meniscus involvement	2 (14%)	2 (14%)
Meniscus Repair	6 (43%)	5 (29%)
Meniscectomy	1 (7%)	3 (21%)

Figure 3.1 CONSORT enrollment flow chart.

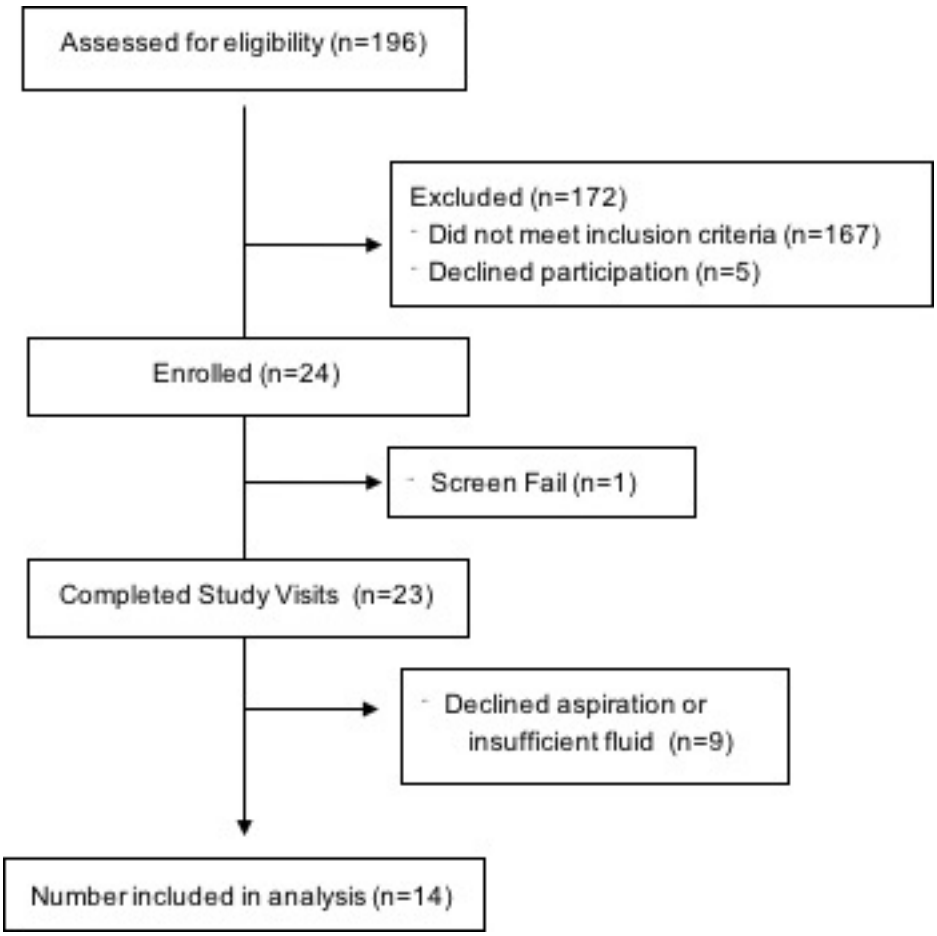
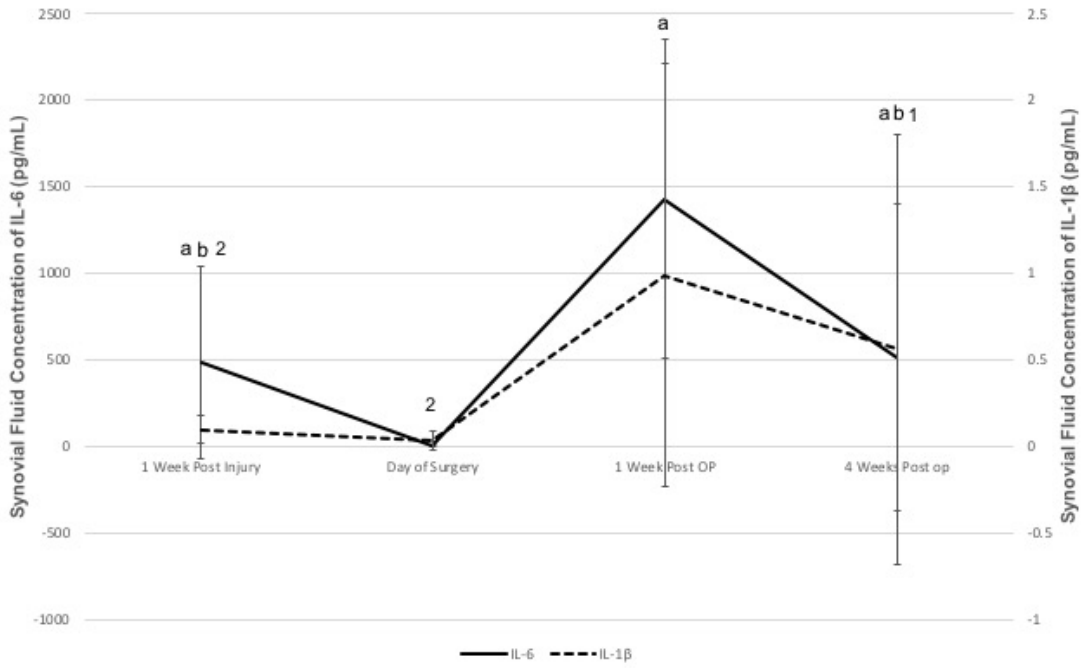


Figure 3.2 Inflammatory progression of IL-1β and IL-6 after ACL injury and reconstruction.

IL-1β concentrations significantly increased one week post-surgery when compared to initial presentation (p=0.015) and the day of surgery (p<0.001)(2). Four weeks post-reconstruction IL-1β concentrations decreased but did not return back to day of surgery levels (p=0.001)(1). There was a significant decrease in IL-6 from the time of initial presentation to surgery (p<0.001)(a). IL-6 concentrations at 1 week post-surgery were significantly greater than initial presentation (p=0.013), the day of surgery (p<0.001), and 4 weeks after surgery (p=0.002)(b). Four weeks post operatively, IL-6 concentrations remained significantly higher than the day of surgery (p<0.001)(a)



"a" Indicates different from the day of surgery p<.001 for IL-6
"b" Indicates different from 1 week post operative p<.05 for IL-6
"1" Indicates different from the day of surgery p=.001 for IL-1β
"2" Indicates different from 1 week post operative p<.02 for IL-1β

CHAPTER 4. UPREGULATION OF SYSTEMIC INFLAMMATORY PATHWAYS FOLLOWING ANTERIOR CRUCIATE LIGAMENT INJURY RELATE TO BOTH CARTILAGE AND MUSCULAR CHANGES: A PILOT STUDY

Abstract

In conjunction with cartilage breakdown, muscle maladaptation including atrophy and increased fibrosis have been observed in the quadriceps following anterior cruciate ligament (ACL) injury. Previously observed upregulated muscle-related proteins in the synovial fluid following ACL rupture allude to cellular communication between the joint and muscle. Therefore, the purpose of this study was to determine whether muscle-related analytes are differentially expressed in the serum. Sixteen patients with an acute ACL tear participated in this IRB-approved study. Serum was obtained at two different time points at a mean 6 and 14 days post injury, and serum was analyzed by a highly multiplexed assay of 1300 proteins. Pathway analysis using DAVID was performed; genes included met three criteria: significant change between the 2 study time points using a paired t-test, significant change between the 2 study time points using a Mann Whitney non-parametric test, and significant Benjamini post hoc analysis. Twelve analytes significantly increased between time points. Proteins chitinase-3-like protein 1 ($p= .01$), insulin-like growth factor binding protein 1 ($p= .01$), insulin-like growth factor binding protein 5 ($p= .02$), renin ($p=.004$) and lymphotoxin alpha1: beta2 ($p= .03$) were significantly upregulated in serum following acute ACL injury. The current results confirm the inflammatory pattern previously seen in the synovial fluid thought to play a role in the progression of post traumatic osteoarthritis after ACL injury, and this data also provides further insights into important communication between the joint and quadriceps group, whose function is important in long term health.

Key Words: Proteomics, ACL, serum, inflammation

Full Reference: Hunt, E. R., Villasanta-Tezanos, A. G., Butterfield, T. A., Lattermann, C., & Jacobs, C. A. (2020). Upregulation of Systemic Inflammatory Pathways Following Anterior Cruciate Ligament Injury Relates to Both Cartilage and Muscular Changes: A Pilot Study. *Journal of Orthopaedic Research®*, *38*(2), 387-392.

Introduction

Traumatic anterior cruciate ligament (ACL) injury causes a deleterious cascade of events including a robust inflammatory response and cartilage breakdown characterized by increases in markers of cytokines and collagen catabolism that could lead to chondrocyte death.[8,12,74,75,107,108] Along with increased pro-inflammatory markers and cartilage breakdown another hallmark of ACL injury is persistent long-term quadriceps atrophy. Atrophy and weakness of this muscle group continues long after injury and even surgical intervention, causing the joint to remain unstable due to a lack of strong dynamic stabilization.[1,3]

Changes in quadriceps morphology post-injury suggest that acute ACL injury affects not only the local environment within the synovial joint but the surrounding structures as well. Following injury, the quadriceps muscle shows increased fibrosis, decreased satellite cell number,[30,31] and a decreased fiber cross sectional area indicating atrophy. Furthermore, after ACL reconstruction, there are upregulations in common muscle growth regulators including myostatin[35] a widely studied signaling molecule of the transforming growth factor-beta superfamily that induces atrophy by activating the ubiquitin proteasome pathway (UPP).[35] What remains unknown is the effect of the inflammatory process on the quadriceps and its influence on changes in muscle. The

differential muscle morphology post injury is relevant because it lends evidence that the joint and muscle may communicate cellularly. This cross-talk between systems could be contributing to the persistent atrophy seen clinically and therefore may identify proteins vital to the interplay between the inflamed joint and quadriceps muscle.

Previously, a study looking at the proteomic response in the synovial fluid to ACL injury revealed upregulated muscle related analytes after injury.[11] Muscle-related analytes myoglobin and SMAD 2/3 are linked to muscle stress and cell growth, but were an unexpected finding in the synovial fluid following acute ACL injury. Therefore, the purpose of this study was to determine whether muscle-related analytes are differentially expressed in the serum. We hypothesized that proteins related to muscle would be increased in the serum after ACL injury, providing a means of transport to the synovial fluid.

Methods

4.1.1 Study Design

This study represents a secondary analysis of samples collected as part of a randomized clinical trial of patients with acute ACL rupture (clinicaltrials.gov: NCT01692756). This RCT aimed to determine the effectiveness of early anti-inflammatory treatment post ACL injury. [12] While the aim of this RCT was to determine the effect of intra-articular triamcinolone acetonide, none of the patients included in this subgroup analysis received a corticosteroid injection at either of the time points used in the current analyses. Subjects included in this proteomic analysis were a part of the placebo group who underwent blood collections via venipuncture, arthrocentesis and

intra-articular saline injections (10 mL). Blood collection was performed on the day of initial presentation (mean= 6 days post-injury, range = 2-8 days) and again between 6 and 12 days after initial presentation (mean=14 days post-injury, range = 8-20 days).

4.1.2 Patient Demographics

This study consisted of 16 patients (8 females, 8 males; mean age 18 years, range 15-26 years; mean body mass index 23, range 18-28 kg/m^2) with acute ACL rupture. Inclusion criteria were as follows: isolated ACL injury determined by clinical exam (positive Lachman test with intra-articular effusion) that was corroborated using magnetic resonance imaging (MRI); age 14 years or older; skeletally mature with closed growth plates visualized by radiograph; no history of ipsilateral knee injury; ACL injury had to occur during sports activities; no clinical evidence of posterior cruciate ligament involvement; and no more than a grade 1 medial or lateral collateral ligament injury. Exclusion criteria were: injury occurrence more than eight days before enrollment; previous knee surgery; history of intra-articular cortisone injection into either knee within three months of injury; or history of any inflammatory disorder.

ACL tear was confirmed intraoperatively at the time of reconstruction and pre-operative MRI ensured there were no concomitant ligamentous injuries. Patients were instructed to avoid any prescription or over the counter anti-inflammatory medications but were allowed to use rest and ice to manually control swelling. All patients received the same rehabilitation protocols which included home based exercises focused on range of motion and quadriceps muscle activation.[109]

4.1.3 Proteomic Analyses

Similar to analyses previously performed on synovial fluid samples,[11] serum samples were collected for proteomic analysis with a high throughput and highly multiplexed assay (SOMAscan version 3, SomaLogic, Inc., Boulder, CO). The technique uses aptamers, chemically modified oligonucleotides that recognize the 3-dimensional structure of proteins with high specificity and high sensitivity. Each aptamer is tagged with a DNA sequence enabling quantification using a hybridization array. The assay converts the measurement of proteins into the measurement of corresponding DNA. Data are recorded for each of the 1317 proteins as relative fluorescent units (RFU). The coefficient of variation for the SOMAscan Assay is 5% (somalogic.com/somascan-assay-faqs).

4.1.4 Statistical Analysis

Paired t-tests were used to determine differences between biomarker levels at the two preoperative time points for each analyte. Due to the small sample size, standardized response means (SRMs) were also calculated to potentially identify any trends within the data. SRM is the pre- to post change divided by the standard deviation of the change scores for that particular variable, and values of >0.8 are considered large.[110,111] In general, higher values of these proteins would reflect a state of greater inflammatory response and a potential propensity for osteoarthritic changes and progression. We also included an exploratory group of five analytes previously found to be upregulated in the synovial fluid that are related to skeletal muscle (myoglobin, SMAD 2/3, CDON, and

fibronectin), three related to inflammation (IL-1β, TGFβ and TNFα) and one related to cartilage breakdown (MMP-1).[11]

The Database for Annotation, Visualization and Integrated Discovery (DAVID Bioinformatics Resources, National Institute of Allergy and Infectious Diseases, NIH) was used to complete the pathway analysis. To be included in the post-hoc pathway analysis, genes had to pass three different statistical tests. All of the genes had to have a significant change between the two study time points using a paired t-test, a significant change between time points using a Mann-Whitney nonparametric test and significant Benjamini post hoc analysis. Upregulated pathways were identified using The Gene Functional Analysis Tool, with Benjamini post hoc analyses to account for multiple comparisons.[112,113] Non-parametric Spearman correlations were used to determine potential relationships between analytes that were significantly upregulated and a known marker of cartilage breakdown (MMP-1). Prior to running the correlations, a Grubbs test for outliers was used to determine the presence of any outliers within the data set. Alpha was set at 0.05 *a priori*.

Results

4.1.5 Analytes with Largest Increase

There were 1,317 analytes measured in total.[11] Of those, 12 demonstrated the largest changes in the serum within the first two weeks after injury (Table 3.1). Even with a small sample size, moderate to large changes in analyte concentrations between time points (SRM ranges -0.58 to 0.69) were observed. There were five analytes that

significantly increased in concentration in the serum within the first two weeks after ACL injury and seven that significantly decreased ($p<0.05$).

A Grubbs test revealed one outlier within the MMP-1 group ($p<0.05$), therefore that subject was excluded from the correlation. There was a moderate correlation (rho =0.64, p = 0.009) between the initial serum levels of Matrix Metallopeptidase 1 (MMP-1) and the change in Insulin Like Growth Factor Binding Protein 5 (IGFBP-5; Figure 3.1).

4.1.6 Pathway Analysis

Four distinct pathways were identified using DAVID analysis. These include complement and coagulation cascades (p=0.003, three analytes identified in this pathway), NF-κβ signaling pathway (p=0.004, three analytes identified), platelet activation (p=0.009, three analytes identified) and ghrelin (p=0.05, two analytes identified). Analytes associated with each specific pathway can be found in Table 3.2.

4.1.7 Analytes Previously Linked to Skeletal Muscle and Inflammation

There were no significant changes in any of the muscle related analytes, inflammatory, or cartilage breakdown markers (Table 3.3). Of these analytes, fibronectin, myoglobin, MMP-1 and IL-1β were decreased whereas the others increased between the two study time points, but not enough to be significant.

Discussion

The purpose of this study was to determine whether muscle-related analytes are differentially expressed in the serum.[11] This would provide a potential mechanism of communication by which proteins may travel to the joint and influence the systemic

environment. In a mouse model of knee OA, increases in inflammatory markers in the serum preceded increases in IGFBP-5 in the muscle and subsequent muscle wasting.[114] Similarly, acute ACL injury initiates a biochemical cascade with increased pro-inflammatory cytokines and increased markers of cartilage breakdown within the joint.[12] Castillero also found increases in inflammatory markers in the serum that preceded increases in IGFBP-5 in the muscle.[114] The muscle related analytes previously found in the synovial fluid[11] were not significantly increased in the serum in the current study, possibly because they are downstream in the signaling pathways. However, IGFBP-5 was elevated in the serum which may have implications for the long-term changes seen within the quadriceps muscle.

4.1.8 Intra-articular Inflammation and Upregulation of IGFBP-5

IGFBP-5 may have important implications for skeletal muscle atrophy after ACL injury. Insulin growth factor (IGF) has important functions throughout the body but most importantly, in this context, it is a potent skeletal muscle growth mediator[115] and may regulate cartilage matrix synthesis.[116] Insulin like binding proteins (IGFBPs) regulate the function of IGF and IGFBP-5 specifically mediates skeletal muscle differentiation and possibly hypertrophy.[115,117,118] IGFBP-5 is the predominatant growth factor binding protein expressed in muscle[119] and can regulate IGF by binding to it with an affinity as high as binding with the IGF receptor. This then allows the binding proteins to influence IGFs[115], either by altering IGF actions[117] or by switching on IGF-II.[120] The regulation of IGFBP-5 is more elusive but studies point to regulation by key micro RNAs[121] like miR-140.[122]

The current study focused on the proteomic analysis after acute ACL injury but there has been some research investigating the role of IGFBP-5 on skeletal muscle and cartilage in induced OA models. Microarray analysis in rats with monoiodoacetate induced OA showed upregulated gene clusters related to skeletal muscle development and specifically increases in IGFBP-5 in animals with cartilage damage.[123] Rats with induced arthritis had significant upregulations in MuRF1, indicating muscle catabolism, in conjunction with increased levels of IGFBP-5 in the gastrocnemius muscle.[124] Induced arthritis via adjuvant injection also showed significant increases in gastrocnemius IGFBP-5 two weeks after injection as well as a significant decrease in gastrocnemius muscle mass and serum IGF-1. Olney et al., found that chondrocytes from osteoarthritic knees had increased expression of IGFBP-5[116], theorizing it may play a role in cartilage breakdown. Our data support this finding and demonstrated a strong correlation between patient's initial levels of MMP-1, a cartilage breakdown marker, and changes in IGFBP-5 (Figure 3.1). These positive correlations suggest a complex communication network that is triggered by intraarticular inflammation and cartilage changes which then result in increased systemic inflammatory markers and finally changes in skeletal muscle.

4.1.9 IGFBP-5 and Muscle Atrophy

In animal models, elevated IGFBP-5 and inhibition of IGF has been consistently associated with atrophy. Stevenson et al. in 2003 found that in atrophied rat soleus IGFBP-5 was elevated four-fold by day four of hindlimb unloading and remained elevated at 14 days.[125] In a study of mouse muscle overload and atrophy, researchers found that the soleus muscle was atrophied by 20% after eight days of unloading, with a two-fold increase in IGFBP-5 and a decrease in IGF-1 mRNA. After eight days of

overload, IGFBP-5 mRNA remained significantly lower in overloaded skeletal muscle than control muscles.[126] Furthermore, overexpression of IGFBP-5 in a mouse model resulted in reduced muscle fiber hypertrophy as well as an overall lower body mass compared to control animals.[118] Steves et al. demonstrated that the tibialis anterior muscles did not grow as expected following reloading after atrophy in IGF over-expressing mice, and reported significantly elevated IGFBP-5 levels three weeks after re-ambulation.[127]

Although quantifying muscle tissue specific IGFBP-5 levels was beyond the scope of this study, our results suggest that IGFBP-5 may be systemically upregulated in first two weeks after ACL injury. Circulating levels of IGFBP-5 presents a potential link between traumatic joint injury and quadriceps atrophy. This finding uncovers an interesting avenue for further investigation of the potential inhibition of IGF in the quadriceps by IGFBP-5 leading to the quadriceps atrophy seen clinically. There are many studies looking at the effects of IGFBP-5 in disuse atrophy and transgenic knockout,[118,120,126,127] but there is little evidence on its effects after traumatic joint injury. A more in-depth exploration into the communication between IGF and IGFBP-5 post ACL injury would be necessary to define its effects on both the joint and musculature. In addition, future studies are necessary to determine if systemic changes in IGFBP-5 also result in changes to not only the quadriceps but other muscle groups as well (hamstrings, gastrocnemius, etc.).

4.1.10 Differences Between Serum and Synovial Fluid Proteomics

Proteomic changes in the serum did not mimic changes in synovial fluid reported previously, revealing fewer and much different analytes and upregulated pathways. While

this was not expected, the upregulated analytes in the serum related to either clotting or inflammation with the exception of the insulin-like growth factor binding proteins. Pathways including complement and coagulation and platelet activation are to be expected due to hemarthrosis into the knee joint after injury.[128] ACL injury creates an influx of pro-inflammatory markers, protein mediators and blood into the joint, which may perpetuate the cartilage damage.[129] Since synovial fluid is an "ultrafiltrate" of plasma and not a static protein pool,[130] contents continuously filter in and out of the joint. Under healthy conditions, there is flow of synovial fluid over the synovial interstitial space that turns over allowing in and out flux of transudate. During periods of trauma the transudate becomes exudate, a protein filled fluid that resides extracellularly in the synovium.[130]

After ACL injury in particular, the length of time that hyaluronan is present in the joint decreases suggesting that there is a transport system for altered synovial fluid post injury.[131] Moreover, the molecular sieve theory postulates that hyaluronan chain length filters the synovial fluid only allowing molecules between 33-59 nm into the articular space[132], thus only allowing certain proteins to flow back and forth. Synovial fluid filtration post injury may be a possible explanation as to why the analytes that were upregulated after injury in the synovial fluid were not the same as those upregulated in the serum. Persistent muscular dysfunction after ACL injury is undoubtedly a complex, multifactorial injury but better understanding the communication between the intra- and extraarticular environments is important in identifying the underlying mechanisms and potential treatment targets.

4.1.11 Limitations

This pilot study of the proteomic response to ACL injury in human serum is not without limitations, particularly that an *a priori* power analysis was not used for sample size. This study included 16 subjects following acute, isolated ACL injury, which limits conclusions that can be drawn and if these results are generalizable to other populations. The progression of inflammation following ACL injury is multifaceted and previous studies show that variables like body mass index, sex and age all effect this progression[133], and should be included in further analysis. These data provide insight into the systemic response after ACL injury and indicate that the effect in serum differs from that in synovial fluid. The present study used samples from two early time points after injury but demonstrates that proteomic analysis can be valuable in determining potential communicating factors between the intra- and extraarticular environments after ACL injury. Future studies focused on the influence of inflammatory and skeletal muscle markers on cartilage is necessary to determine the interplay between the local joint environment and surrounding structures.

Conclusions

ACL injury affects not only the knee joint but the surrounding musculature as well. The current proteomic analyses identified analytes that could have implications for inflammation, muscle atrophy, and cartilage health. The proteomic analysis of the serum provided insight into systemically upregulated analytes that could be important for communication between the inflamed joint and subsequent changes in muscle. Identifying key players in the communication between the intra- and extraarticular

environments will be crucial to developing successful interventions for the prevention of muscle atrophy and cartilage breakdown. Further investigations will be necessary to elucidate the relationship of analytes like IGFBP-5, inflammation, and muscular changes after ACL injury.

Table 4.1 Twelve analytes that were significantly up or down regulated between the time of ACL injury and one-week post injury.
All values are expressed in relative fluorescent units (RFU; mean ± standard deviation).

Protein	***Time 1***	***Time 2***	***P***	***SRM***
Chitinase-3-like protein 1	5468.9 ± 3369.2	8102.8 ± 6508.8	0.015	0.69
Insulin-like growth factor-binding protein 5	1697.6 ± 323.04	1992.9 ± 531.9	0.015	0.69
Insulin-like growth factor- binding protein 1	659.6 ± 389.9	1070.6 ± 816.6	0.030	0.60
Renin	368.0 ± 109.6	435.1 ± 96.3	0.032	0.59
Lymphotoxin alpha1: beta2	193.2 ± 52.8	238.3 ± 96.7	0.038	0.57
Cathepsin Z	3006.1 ± 751.1	2497.4 ± 778.8	0.034	-0.58
Fibrinogen	32497.3 ± 12149.5	25956.5 ± 7428.3	0.024	-0.63
Carbonic anhydrase 6	10018.4 ± 4794.5	8828.9 ± 4425.8	0.005	-0.63
Fibrinogen gamma chain	7449.6 ± 2530.2	5850.8 ± 1547.8	0.020	-0.65
Lipopolysaccharide-binding protein	45316.3 ± 10568.0	36271.2 ± 12465.6	0.015	-0.69

Table 4.1(continued)

C-reactive protein	**11677.4 ± 12578.4**	**5246.2 ± 6217.1**	**0.012**	**-0.71**
Parathyroid hormone	1169.3 ± 317.3	961.7 ± 226.7	0.007	-0.79

Table 4.2 Analytes identified for each of the significant pathways

Pathway	***Analytes***
Complement and coagulation cascades	Fibrinogen alpha chain (FGA)
	Fibrinogen beta chain (FGB)
	Fibrinogen gamma chain (FGG)
NF-kappaβ	Lipopolysaccharide binding protein (LBP)
	Lymphotoxin alpha (LTA)
	Lymphotoxin beta (LTB)
Platelet activation	Fibrinogen alpha chain (FGA)
	Fibrinogen beta chain (FGB)
	Fibrinogen gamma chain (FGG)
Ghrelin	Insulin like growth factor binding protein 1 (IGFBP1)
	Insulin like growth factor binding protein 5 (IGFBP5)

Table 4.3 Muscle, inflammatory and cartilage related analytes between the time of injury and one-week post injury.

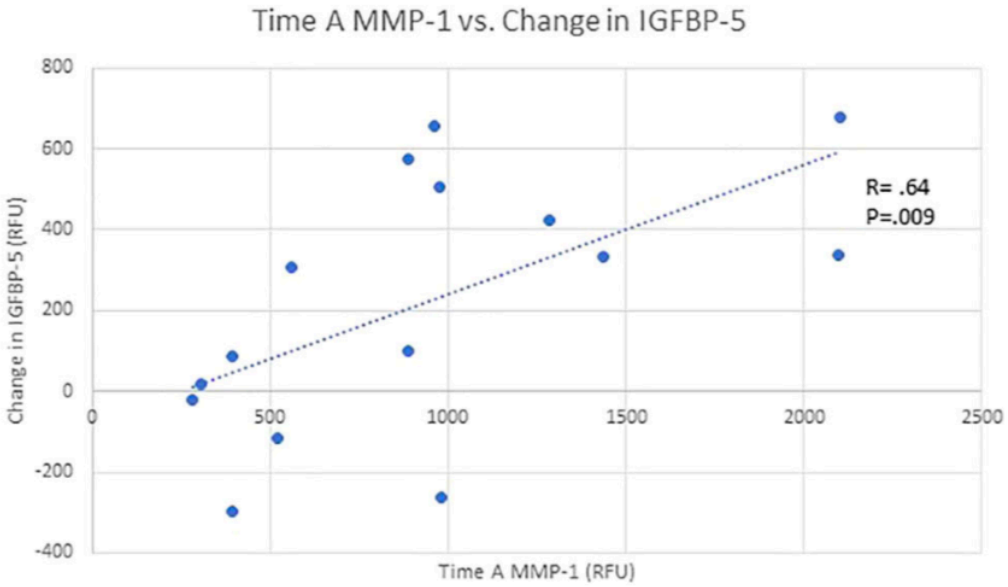
All values are expressed in relative fluorescent units (RFU; mean ± standard deviation).

Muscle Related Analytes	***Time 1***	***Time 2***	***P***	***SRM***
Cell adhesion molecule-related/down-regulated by oncogenes	5028.8 ± 1293.7	5240.1 ± 1323.0	0.42	0.21
Fibronectin	11152.4 ± 4289.1	8621.0 ± 5605.3	0.15	-.38
Myoglobin	1046.1 ± 368.8	889.1 ± 405.5	0.31	-0.26
Mothers against decapentaplegic homolog 2	983.3 ± 479.6	8628.3 ± 27301.5	0.28	0.28
Mothers against decapentaplegic homolog 3	2997.6 ± 1625.0	3258.0 ± 3316.6	0.76	0.08

Table 4.3 (continued)

Inflammatory Related Analytes	*Time 1*	*Time 2*	*P*	*SRM*
Interlukin 1 Beta	1129.1 ± 571.	918.9 ± 491.7	.20	-0.33
Transforming growth factor beta-1	1509.0 ± 929.3	1810.7 ± 1707.0	0.46	0.19
Tumor necrosis factor alpha	388.9 ± 208.3	470.4 ± 422.4	0.38	0.23
Cartilage Related Analytes	***Time 1***	***Time 2***	***P***	***SRM***
Interstitial collagenase	937.2 ± 586.9	113.4 ± 977.2	0.67	-0.11

Figure 4.1 Scatter plot depicting a significant (p=.009) positive linear relationship between time A matrix metallopeptidase 1 (MMP-1) and the change in insulin like growth factor binding protein 5 (IGFBP5).



CHAPTER 5. PHYSIOLOGICAL CHANGES TO THE VASTUS LATERALIS AFTER NON-INVASIVE ANTERIOR CRUCIATE LIGAMENT INJURY

Abstract

Insufficient recovery of quadriceps muscle strength is commonly reported after anterior cruciate ligament (ACL) injury. Although weakness is secondary to a complex manifestation of neural inhibition, the extent and time course of morphological changes in muscle are largely unknown. Using a novel, clinically relevant animal model of ACL injury, a longitudinal study was performed to illuminate mechanisms underlying muscle atrophy. **Purpose** To investigate changes in cellular parameters underlying muscle function after non-invasive ACL rupture. **Methods** Male Long-Evans rats were randomly assigned to 8 groups (n=6-8 per group): one control group and seven groups after ACL injury (time after injury: 6, 12, 24, 48-hours, and 1, 2, 4-weeks). The right hindlimb of rats in the ACL-injury group were exposed to a single load of tibial compression to induce a non-invasive ACL rupture, followed by normal cage activity for designated time. The vastus lateralis (VL) muscles were immunoreacted for IgG, Pax7 and dystrophin to quantify muscle damage, satellite cell number and muscle fiber cross sectional area (CSA) respectively. The mRNA abundance of muscle RING-finger protein-1 (MuRF-1), muscle atrophy F-box (MAFbx) (markers of protein degradation), 45s pre-rRNA (marker of ribosome biogenesis) and CD68 (marker for macrophage abundance) was determined by RT-PCR. One-way ANOVAs with Bonferroni post-hoc were used to determine differences between groups and paired t-tests were used to detect differences between limbs ($P < 0.05$). **Results** ACL injury resulted in a decrease in muscle wet weight ($p=0.0003$) and a trend toward reduced CSA ($p=0.06$) at 1-week post injury only, compared to control. CSA of the ACL injured limb was smaller than that of

the contralateral limb at 1-week only (p= 0.01). MAFbx mRNA abundance was significantly increased at 48-hours post-ACL injury (p=0.0001), with no differences for IgG, central nuclei, Pax7, 45s rRNA, total RNA concentration, CD68 or MuRF-1. **Conclusions** Results indicate that ACL injury induces atrophy which is transient and not related to muscle damage or changes in ribosome biogenesis but could be due to increased protein degradation. Based on these analyses future studies should focus on corroborating the increase in atrophic pathways and determining the presence of protein breakdown after ACL injury, to establish key therapeutic windows for targeting therapy-resistant quadriceps weakness after ACL injury. Supported by K01AR071503.

Keywords: Anterior cruciate ligament, quadriceps, atrophy

Introduction

Anterior cruciate ligament (ACL) injuries are one of the most common musculoskeletal injuries with an incidence rate of upwards of 200,000 cases a year.[1] One detrimental result of ACL injury is persistent long term quadriceps atrophy and weakness.[134-138] While the current standard treatment for ACL injury is surgical reconstruction, surgery does not entirely solve the problem of muscle atrophy.[139] Reconstruction surgery will reestablish joint congruity but patients who undergo surgery and those who remain ACL deficient will have quadriceps atrophy and weakness. [23,24,140] ACL reconstruction and non-surgical treatment yield similar long term outcomes but when compared to the contralateral leg or healthy controls, patients who have town their ACL still have quadriceps atrophy and weakness.[26] Furthermore, the incidence of developing post traumatic osteoarthritis (PTOA) is similar for patients who undergo

reconstruction as for those who remain ACL deficient (ACLD), indicating a need for interventions that will help preserve long term joint health. Patients with decreased quadriceps muscle strength have a higher incidence of PTOA likely due to the quadriceps' inability to mitigate force through the joint. Therefore, elucidating the sources of quadriceps weakness following ACL injury is imperative in developing proper interventions.

Quadriceps atrophy following ACL injury is multifactorial including alterations in neural inhibition[17-19] and muscle morphology[30,31,33]. In comparison to what is known about alterations in neural inhibition, less is known about the cellular responses of the quadriceps muscle itself. Following ACL injury there are decreases in both physiological cross sectional area and fiber cross sectional area, with the largest decreases in the vastus lateralis between injured and non-injured limbs.[29,30] There are also abundant increases in collagen content and fibrosis following injury[31,33] that is associated with decline in satellite cell abundance.[30,31] This could have important implications for function because increases in fibrosis and a decreased muscle stem cell abundance could limit muscle hypertrophy and muscle regrowth[31]. Additionally, there is a rise in myostatin expression in both the muscle and circulating serum levels[33,35].Myostatin is an important muscle growth mediator[34] and could be involved in atrophy following injury. Furthermore, studies looking at human muscle biopsies following ACL injury show higher levels of inflammatory mediators like TNFα and IL-6[33], presenting an inflammatory response in the muscle following joint injury. These studies provide an important first step in understanding the response of the quadriceps muscle following injury. Studies involving human subjects are mostly cross sectional and due to the variability and constraints of

human research, these studies are confounded by long periods between injury and biopsy as well as reconstruction surgery. Therefore, there is a need for longitudinal studies that will provide insight into timing and mechanisms underlying vastus lateralis atrophy after ACL injury.

Pre-clinical animal models of ACL transection provide a more controlled way to study the effects of ACL injury. The use of animal models allows researchers to study time points, like acutely after injury, that would not be feasible in human studies. However, transection surgery introduces more confounding variables similar to reconstruction surgery like an increase in inflammation and upregulation in certain atrophic markers.[36,37,41] A non-invasive model of ACL injury would allow for a translational approach to determining the mechanisms responsible for muscle atrophy. Therefore, the purpose of this study was to use our in vivo pre-clinical model of ACL injury to systematically study the quadriceps muscle at several time points after injury. We hypothesized that following ACL injury, atrophy would set in early after injury, preceded by damage and inflammation in the muscle, and would continue for the duration of the study, up to four weeks after injury.

Methods

5.1.1 Animals

All procedures performed were approved by the University of Connecticut's Institutional Animal Care and Use Committee. Male Long Evans rats, aged 16 weeks, were used for this study. Animals were housed at the University of Connecticut division of animal research facility. Rats were kept in normal sized cages on a 12:12 light/dark

cycle and had access to food and water ad libitum. Rats were randomly divided into seven experimental groups (6 hr (n=6), 12 hr (n=6), 24 hr (n=6), 48 hr (n=6), 1 wk (n=8), 2 wk (n=8), 4 wk(n=8)) and one sham uninjured group (n=6). The right hindlimb was subject to ACL tear and after recovery animals were euthanized corresponding to the group time point.

5.1.2 ACL Tear

Upon arriving at the vivarium, rats underwent a one week acclimatization period. Rats that were randomly assigned to the ACL injury group, underwent a reproducible non- invasive ACL injury using a single load of tibial compression. The custom device consists of two custom-built loading platforms. 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 30 deg of dorsiflexion and 100 deg 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 are anesthetized using an induction chamber with 5% isoflurane and 1L/min oxygen and maintained via a nose cone with 2% isoflurane and 500mL. The right hindlimb is subjected to single load of tibial compression at a speed of 8mm/s. ACL injury is noted by a release of compressive force during injury that was monitored via a custom program (LabVIEW, National Instruments, Austin, TX). Post-injury, study personnel perform a Lachman's test to clinically confirm an ACL rupture has occurred and the hindlimb was palpated to check for the presence of gross bone deformity. If no contraindications were present, rats were returned to their cages and allowed to recover, no opioids or analgesics were administered due the ability for these drugs to alter normal

biologic responses. Following euthanasia rats were weighed and bilateral vastus lateralis muscles were harvested, weighed, flash frozen and stored at -80 degrees for further analysis. The right hindlimb was used for all analyses, except for CSA where both hindlimbs were utilized.

5.1.3 Immunohistochemistry

IgG. To determine whether muscle fibers from the vastus lateralis exhibited overt muscle damage, fibers were immunoreacted for immunoglobulin G (IgG) infiltration[141,142]. Sections (8μm) were fixed in ice cold acetone (100%) for 10 minutes and washed in phosphate buffered saline (PBS). Slides were then incubated in fluorescein isothiocyanate-conjugated mouse anti-IgG (Invitrogen, Carlsbad, CA) overnight at 4°C. Following incubation, slides were washed twice in 5% bovine serum albumin and then once in PBS. To finish, sections were coverslipped using Vectashield mounting medium (H-1000, Vector Laboratories, Burlingame, CA). Images were captured using a Zeiss Axio Imager M1 microscope (Carl Zeiss, Göttingen, Germany). Five fields, taken to reflect the non-homogenous nature of the muscle, to ensure that all areas of the muscle were represented were photographed (100x) and subsequently analyzed using ZEN blue software (Carl Zeiss). Fluorescent intensity was quantified by the densitometric mean of each fiber (AU, arbitrary units) as previously described.[142] IgG density, a marker of overt fiber damage was counted by a trained-blinded assessor. **Pax7**. To quantify the abundance of Pax7+ cells (satellite cells)[142,143], slides were fixed in 4% paraformaldehyde and underwent antigen retrieval at 92°C using sodium citrate buffer. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide and then sections were incubated in Pax7 primary antibody (Developmental Studies Hybridoma Study Bank,

Iowa City, IA, USA) at a 1:100 dilution. Next muscle sections were immunoreacted with biotin conjugated secondary antibody (1:1000) (Jackson ImmunoReseach Laboratories, West Grove, PA, USA) and then streptavidin-horseradish peroxidase to amplify. Sections were then reacted with using TSA-Alexa fluor 488 (Invitrogen, Carlsbad, CA) and stained with DAPI to visualize nuclei. Pax7+/DAPI+ nuclei were expressed per number of muscle fibers. **Dystrophin/Central Nuclei**. Mean fiber cross sectional area (CSA) was determined by immunoreacting muscle sections with dystrophin (Thermo Fischer Scientific, Waltham, MA) primary antibody for 1 hour at room temperature and then overnight at 4°C. Sections were incubated with directly conjugated goat-anti mouse 488 secondary antibody. Sections were also immunoreacted with DAPI and then cover slipped with Vectashield mounting medium. Images were captured using Zeiss Axiovision software and Myovision analysis software[144] was used to cculate mean fiber cross sectional area of each of the muscle fibers. Central nuclei were determined by counting all DAPI+ positive nuclei with clear separation from the dystrophin border by a blinded trained assessor[145].

5.1.4 RNA Isolation and Real Time RT-PCR

RNA isolation was performed briefly as follows[143,146]. VL tissue was homogenized using a stick homogenizer in 1 mL of Trizol (Invitrogen, Carlsbad, CA). Homogenized muscle was spun at 12,000 g for 15 minutes and the supernatant was collected and added to 200 µl chloroform. Tubes spun again at 12,000 for 15 minutes and the aqueous layer was taken for precipitation. Once precipitation occurred, the pellet was washed and reconstituted using RNAse free water. RNA concentration was measured using a Nano Drop (Thermo Fisher Scientific, Foster City, CA). Quantitative real-time reverse

transcription polymerase chain reaction (RT-PCR) was preformed using chemistry, protocol and the amplification and detection of Applied Biosystems (Thermo Fisher Scientific, Foster City, CA). For each sample cDNA was synthesized from 1 μg of total RNA using iScript Reverse Transcriptase according to manufacturer's protocol (Biorad, Hercules, CA). Primers were selected using the Primer Design function of the Primer Express v1.5 software (Applied Biosystems, Foster City, CA) and were as follows: 18s (M11188) forward, TTCGGACGTCTGCCCTATCAA, reverse, ATGGTAGGCACGGCGACTA; tubulin (NM 022298) forward, GGCATGGAGGAGGGAGAGTT, reverse, CCAACCTCCTCATAATCCTTCTCTAG; β2-microglobulin (NM 012512.2) forward CGTGCTTG CCATTCAGAAAA, reverse GAAGTTGGGCTTCCCA TTCTC; muscle atrophy F-box (MAFbx) (NM 133521) forward, GACCTGCATGTGCTCAGTGAA, reverse, GGATCTGCCGCTCTGAGAAGT; muscle RING finger protein-1 (MURF1) forward, TGCCCTGCCAGCACAAC, reverse, GGATTGGCAGCCTGGAAGAT; 45s forward, GAACGGTGGTGTGTCGTT, reverse, GCGTCTCGTCTCGTCTCACT; CD 68, forward, TGTCTGATCTTGCTAGGACCG, reverse, GAGAGTAACGGCCTTTTTGTGA. PCR reactions for 18s, β2-microglobulin, tubulin, MuRF1, MaFbx, 45s and CD68 were assemble using the SYBER Green PCT Master Mix, which only required the addition of cDNA template and the primers mentioned above. Data points for the standard curve were generated using threefold serial dilutions of pooled cDNA. The reactions were performed using ABI PrimsTM 7700 Sequence Detection System (Applied Biosystems) and the systems universal cycling conditions.

RNA abundance was expressed a ratio that was normalized to the Geomean (Tubulin, 18s and β2-microglobulin).[143,146]

5.1.5 Statistical Analysis

Using PRISM 8 software, one-way ANOVA with a Dunnett's test was used to determine significance between ACL-injured groups and control. For within group comparisons, paired t-tests were utilized. All values reported are mean ± standard error of the mean (SEM) and statistical significance was assumed at $P<0.05$.

Results

There were significant differences in VL wet weight with the one-week time point being lower compared to control ($p=0.001$) and muscle weight to body weight ratios at the one-week ($p=0.001$) and two-week ($p=0.025$) time points being lower compared to the control group (Figure 5.1). Muscle fiber CSA was lower and trending towards significance at the one-week time point ($p=0.06$) compared to control. To confirm that this muscle atrophy seen at one week was due to the injury and not the animals losing weight we compared fiber CSA of the left (non-injured) and right (injured limbs). The right limb at the one-week time point was significantly decreased compared to left ($p=0.011$) (Figure 5.2).

There were no differences in IgG infiltration between any of the experimental groups compared to control ($p=0.310$), indicating that the VL muscle did not undergo overt muscle damage in response to the ACL injury (Figure 5.3). There were also no differences in the number of central nuclei per fiber ($p=0.690$) (Figure 5.4). CD68, macrophage marker, mRNA abundance was not different between any of the

experimental groups and control (p=0.0363). mRNA abundance of TNFα, IL6 and IL10 was not detectable in our tissues (Figure 5.5). There were no differences in Pax7+ satellite cells abundance between the groups (p=0.210) (Figure 5.6).

Next we looked at specific atrophic markers, MuRF1 and Mafbx and found that Mafbx at the 48 hour time point (p=0.004) was significantly higher compared to control (Figure 5.7), but MurF1 was not different (p=0.296) at any time point. There were no significant differences in RNA concentration (p=0.221) or 45s pre-ribosomal RNA (p=0.368) (Figure 5.8).

Discussion

Results from this study show that following ACL injury atrophy of the quadriceps muscle begins as early as 1 week post injury. In our model we found fiber atrophy at one-week that was preceded by increases in an atrophic marker at 48 hours post injury. These data parallel previous longitudinal studies[36,41] which showed increases in MaFbx and Murf1 three days post ACL transection surgery. This is important because it provide insights into the period when atrophy is beginning which may have implications in developing interventions to treat muscle atrophy following ACL rupture. The model we utilized here is fundamentally different from other pre-clinical models considering the mode in which injury was induced. Surgical ACL transection models confound the data because opening the joint during a transection procedure causes an increases in inflammation and upregulations in atrophic markers, in both the ACL transection group and the sham surgery group.[36,41] Increases in protein degradation markers would imply that the atrophy seen in the quadriceps is a function of the surgery and not the injury.[36] Our model is a

closed ACL injury, meaning we induce ACL injury without opening the knee joint. Therefore, the decreases we see in muscle size and increases in atrophic markers demonstrates that these processes are in fact caused by injury to the ligament and will happen regardless of surgery.

In conjunction with looking at markers of atrophy we also looked at RNA concentration, an indicator of ribosome quantity or translational capacity, and pre-ribosomal RNA, an indicator of RNA transcription. Increases in pre-ribosomal RNA may indicate an increased capacity for ribosome biogenesis. While this is not a direct measure of protein synthesis, these markers could begin to discern changes in a cell's capacity for protein synthesis.[147] While our data did not find any differences in RNA concentration or 45s pre-rRNA, we did see a rebound in muscle size. CSA was increased back to control level by two weeks, which could be due to the animals using their hindlimb more normally as they recover from injury but remains different from how humans respond to this injury, who do not recover muscle size. Animals in this study were allowed to use their limb unrestricted after ACL injury which is a departure from how human patients are treated; they are traditionally put in a brace following ligament injury. Bracing does not aid in restoring normal biomechanics or function to the joint[148] and immobilization of the knee may aid in quadriceps atrophy.[149] There is evidence to support that rehabilitation preceding ACL reconstruction is helpful in restoring patient function after surgery[150,151] but elucidating the timing of when atrophy begins, will help clinicians determine the most appropriate treatment immediately following injury. Furthermore, few studies have investigated changes in protein synthesis following ACL injury, but the interplay between

protein synthesis and degradation is important and futures studies need to determine how protein synthesis is changing in response to injury and an increase in atrophy.

This was one of the first study to investigate markers of muscle damage in the vastus lateralis following ACL injury. Along with IgG infiltration, to confirm the absence of overt injury, we counted central nuclei, and satellite cells which are necessary for muscle regeneration[152], and also saw no differences in these markers in any of the experimental groups. The lack of damage insides fibers in our tissues demonstrates that our model was successful in administering a joint injury without disruption to the surrounding musculature and bone. This indicates that the atrophy we see at one week is due to the effects of ligament rupture and not a response to damaging stimulus. Moreover, we also found no differences in CD68 RNA indicating there were no differences in macrophage abundance between groups in the muscle. The lack of inflammatory markers in our muscle samples is a departure from what is seen in the human studies where inflammation has been detected in the vastus lateralis muscle[33] and serum.[35] Differences in the inflammatory response following ACL injury could be due to the length of time between injury and biopsy or the absence of surgical intervention in our model. Human studies looking at the effects of ACL injury on quadriceps muscle are limited by the timeframe in which they can collect samples from their patients and on average the time between injury and muscle collection is almost three months.[30,31,33] It is likely that we did not find the same response in the muscle as previous human literature because our model was looking at acute post-injury time points and the response of the muscle may be different early after injury. In the time between injury and sample collection there is the possibility that patients could have been exercising or involved in

rehabilitation that may increase inflammatory markers in the muscle and serum.[153,154] This could explain the differences between the inflammatory response seen in the muscle of human subjects in previous studies and our model presented here.

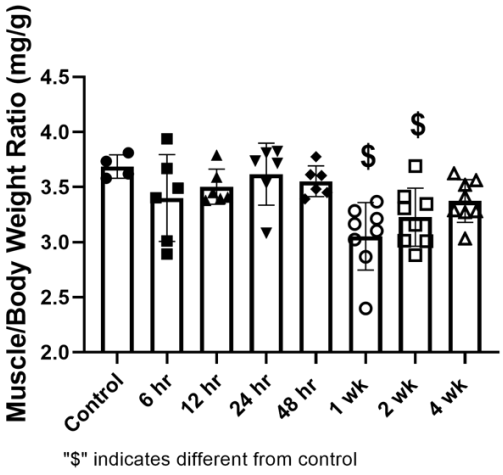
This study is not without limitations as this was the first step in beginning to understand how the quadriceps responds to closed ACL injury in the early acute phase after injury. Our latest time point was four weeks post injury and based on human literature it is likely that there may be alterations to the quadriceps as far out as three years after injury. We also did not include a sham treatment group in our studies to see how positioning the animal's hindlimb into the apparatus only without the injury, would affect the muscle. Lastly, we did not look at any markers of fibrosis or protein breakdown and in the future, these would be important additions.

Conclusions

This study was an important first step in understanding how ACL injury affects the quadriceps in the acute injury phase. This study utilized a novel device to induce a closed ACL tear and longitudinally studied the quadriceps muscle physiology over time. There was no overt damage or inflammation to the muscle but there were upregulations in atrophic markers at 48 hours and fiber atrophy at 1 week that may be important in developing and implementing interventions to combat the muscle atrophy seen clinically. Future studies should focus on continuing to elucidate the mechanisms behind quadriceps atrophy in response to ACL injury.

Figure 5.1 Muscle weight is lower at 1 week post ACL injury indicating atrophy in the vastus lateralis.

Differences in muscle wet weight between time points, "$" indicated that one week is different from control (p=0.001). Significant decreases in muscle weight to body weight ratio for the 1 week (p=0.001) and 2 week (p=0.025) time points compared to control (con (n=6), 6 hr (n=6), 12 hr (n=6), 2 hr (n=6), 48 hr (n=6), 1 wk (n=8), 2 wk (n=8), 4 wk (n=8)). All values reported are mean ± standard error of the mean (SEM).



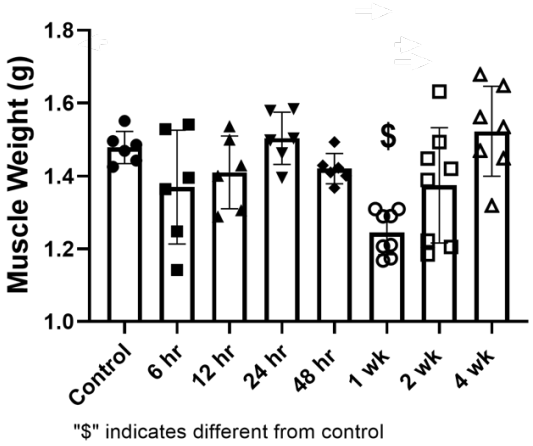


Figure 5.2 Vastus Lateralis atrophy at 1 week following ACL injury.

Representative pictures of vastus lateralis cross-sectional area stained with dystrophin (green) for control and 1 week groups. Decreases in fiber CSA at the 1 week time point trending towards significance (p=0.06). Significant decreases in fiber CSA in the right limb compared to the left limb for the one 1 week animals (p=0.011. Con (n=6), 6 hr (n=6), 12 hr (n=6), 2 hr (n=6), 48 hr (n=6), 1 wk (n=8), 2 wk (n=8), 4 wk (n=8). All values reported are mean ± standard error of the mean (SEM).

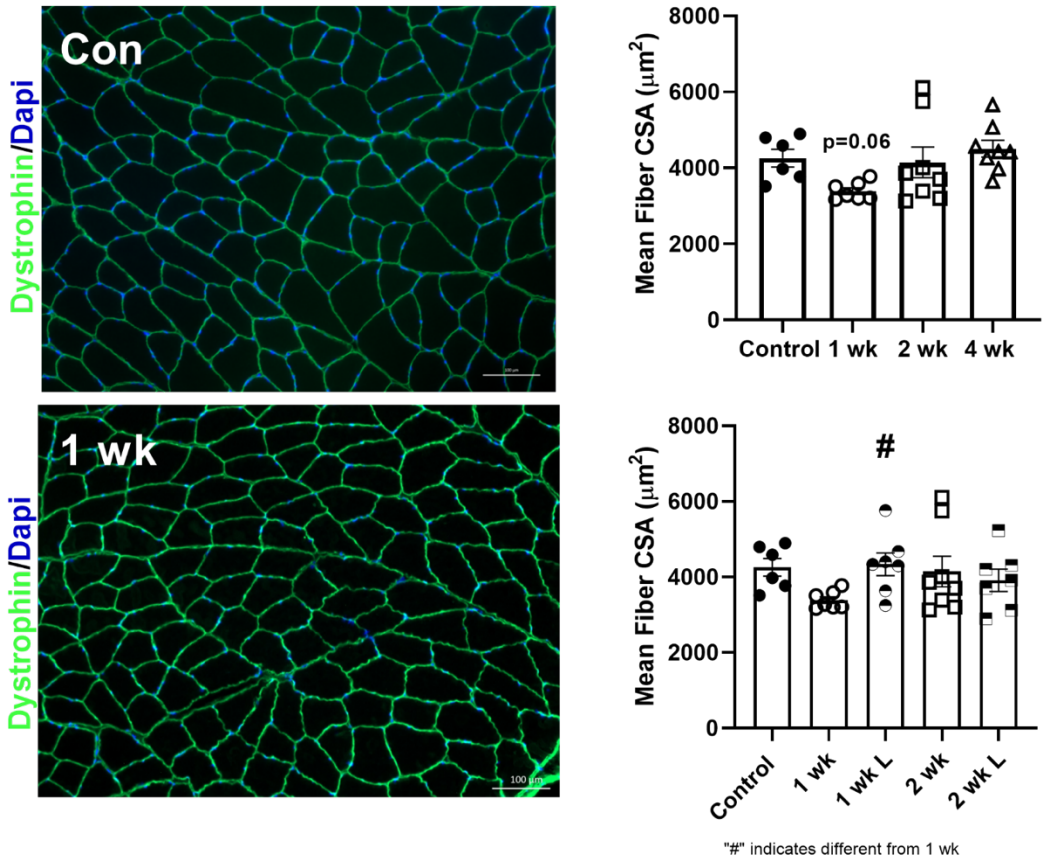


Figure 5.3 ACL injury does not cause overt muscle damage.

Representative pictures of IgG photos from a control and 6-hour animal. No differences in IgG density between any of the time points (con (n=4), 6 hr (n=6), 12 hr (n=6), 2 hr (n=6), 48 hr (n=6), 1 wk (n=8), 2 wk (n=8), 4 wk (n=8)) and control (p=0.310). All values reported are mean ± standard error of the mean (SEM).

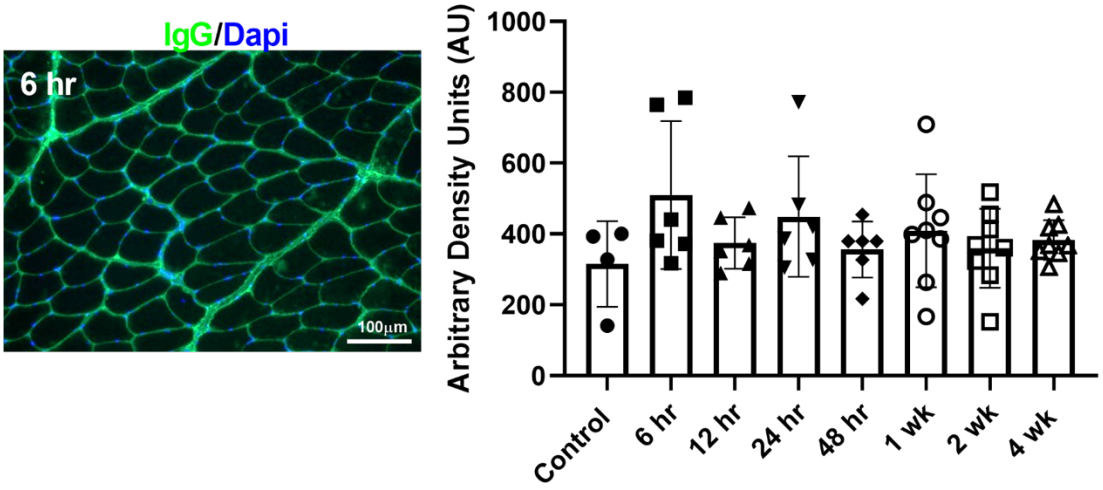


Figure 5.4 ACL injury does not increase the number of central nuclei.

Representative images of central nuclei from a control and 48 hour animal. No differences in the number of central nuclei per fiber between any time point (con (n=4), 6 hr (n=6), 12 hr (n=6), 2 hr (n=6), 48 hr (n=6), 1 wk (n=7), 2 wk (n=8), 4 wk (n=8)) and control (p=0.690). All values reported are mean ± standard error of the mean (SEM).

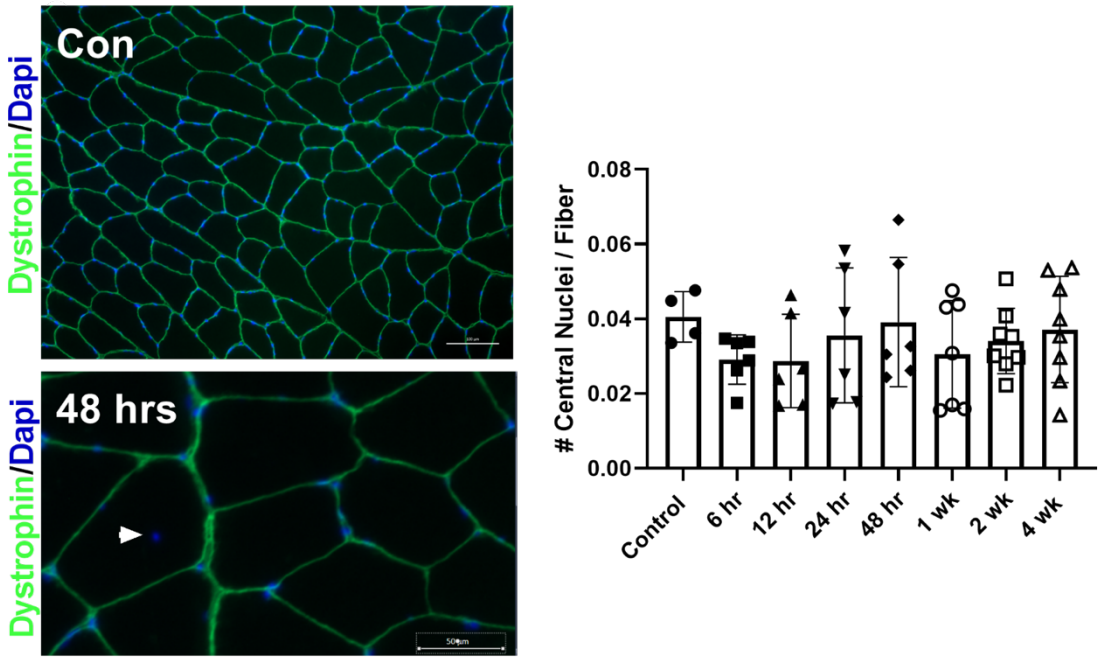


Figure 5.5 No differences in CD68 mRNA abundance after ACL injury.

There were no differences in CD 68 gene expression in the injured limb between any of the time point (con (n=6), 6 hr (n=4), 12 hr (n=6), 2 hr (n=6), 48 hr (n=5), 1 wk (n=7), 2 wk (n=8), 4 wk (n=6)) compared to control (p=0.363). All values reported are mean ± standard error of the mean (SEM).

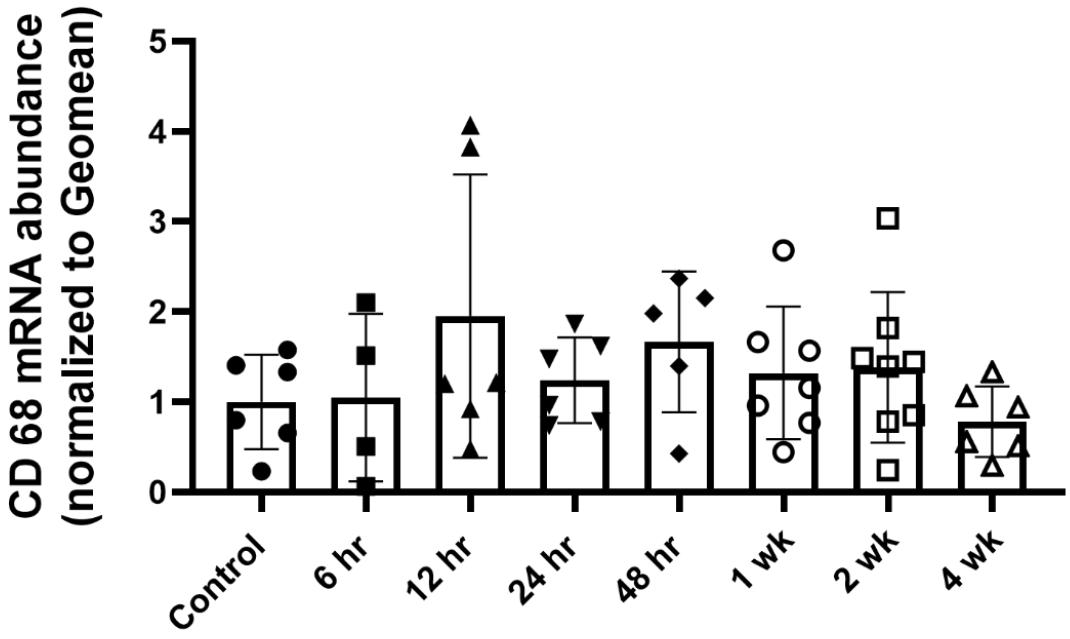


Figure 5.6 No differences in Pax7+ cells after ACL injury.

Representative photos of Pax7+ cells (green) from control and 12-hour groups. There were no differences between Pax7+ cells per fiber between any of the time point (con (n=6), 6 hr (n=6), 12 hr (n=6), 2 hr (n=6), 48 hr (n=6), 1 wk (n=8)) compared to control (p=0.210). All values reported are mean ± standard error of the mean (SEM).

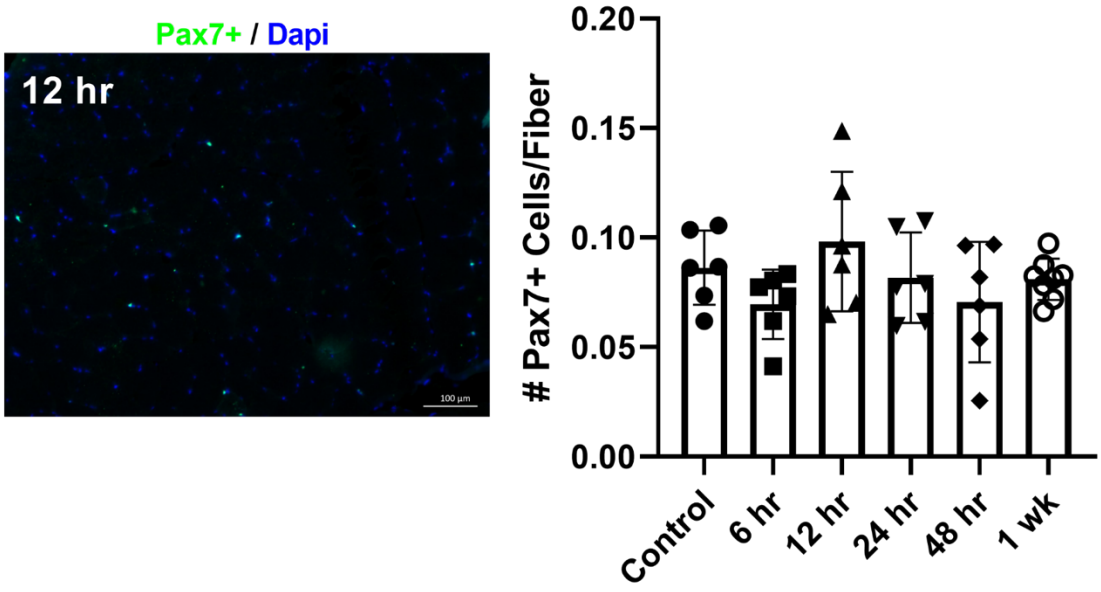
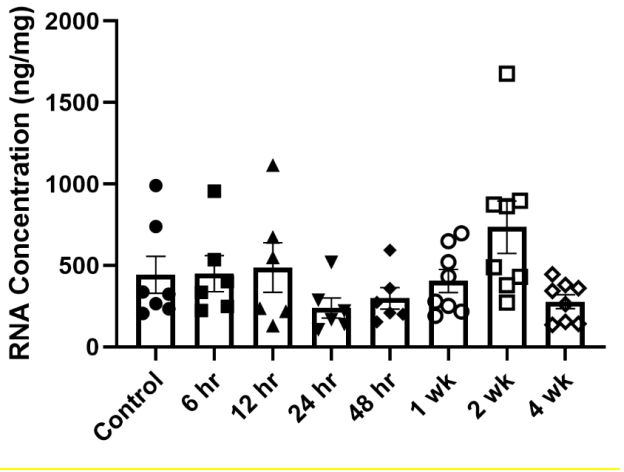


Figure 5.7 No differences in RNA concentration or pre-ribosomal RNA after ACL injury.

RNA concentration is not different between control (con (n=7), 6 hr (n=6), 12 hr (n=6), 24 hr (n=6), 48 hr (n=6), 1 wk (n=8), 2 wk (n=8), 4 wk (n=8)) and any other time point (p=0.221). There is no difference in 45s gene expression between control and every other time point (p=0.368). All values reported are mean ± standard error of the mean (SEM).



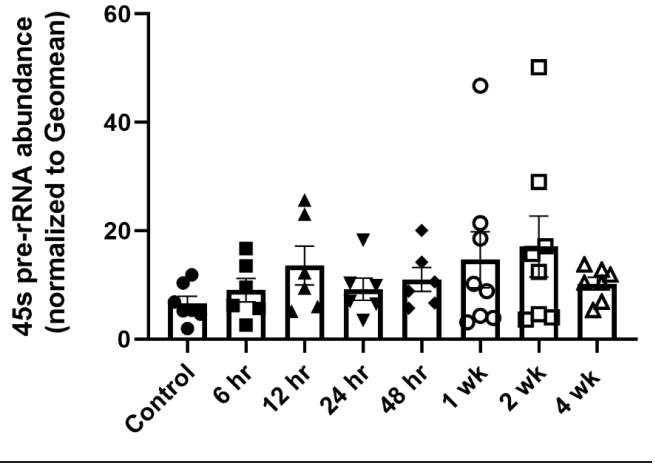
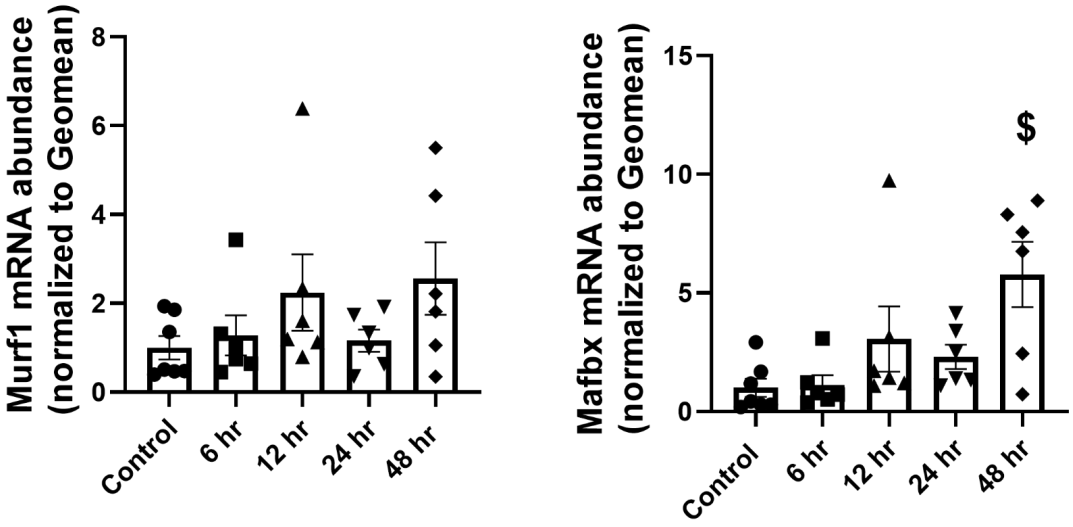


Figure 5.8 Difference in Murf-1 and Mafbx gene expression after ACL injury.

Murf-1 gene expression is not different between control (con (n=7), 6 hr (n=6), 12 hr (n=6), 2 hr (n=6), 48 hr (n=6), 1 wk (n=8), 2 wk (n=8), 4 wk (n=8)) and any other time points (p=0.296). Mafbx gene expression is significantly higher at 48 hours when compared to control (p=0.004). All values reported are mean ± standard error of the mean (SEM).



CHAPTER 6. SUMMARY

Purposes, Aims, and Hypotheses

The purpose of this dissertation is twofold: Firstly, to examine the relationship between serum, synovial joint inflammatory markers and proteomics in ACL deficient subjects; and 2. use those acquired data to identify cellular processes to track longitudinally in conjunction with changes in muscle size following a closed ACL injury in a rat model. These studies were designed to address the following aims and hypotheses:

1. To determine if patients who remain ACLD have atrophy and weakness of the of the quadriceps compared to patients who undergo reconstruction.

   Hypothesis: Patients who remain ACLD will have similar functional outcomes as those who underwent surgery for isokinetic muscle strength, the single leg hop test and the IKDC.

2. To assess inflammatory biomarkers before and after ACL reconstruction to determine if surgery results in a secondary inflammatory response.

   Hypothesis: We hypothesized that markers of inflammation (interleukin-1β, (IL-1β), interleukin-6 (IL-6)) are significantly increased following ACL reconstruction surgery.

3. To determine whether muscle-related analytes are differentially expressed in the serum.

   Hypothesis: We hypothesized that proteins related to muscle would be increased in the serum after ACL injury, providing a means of transport to the synovial fluid.

4. To use our in vivo pre-clinical model of ACL injury to systematically study the quadriceps muscle at acute time points after injury

   Hypothesis: following ACL injury there would be cellular changes, such as damage, inflammation and atrophy, to the vastus lateralis muscle.

Summary of Findings

The summary of findings for each specific aim are presented below. The findings include the following:

1. To determine if patients who remain anterior cruciate ligament deficient (ACLD) have atrophy and weakness of the of the quadriceps compared to patients who undergo reconstruction.

   Findings: The findings of this CAT imply that non-operative treatments of ACL injuries are adequate options in restoration of patient function, as well as patient strength at long term follow ups. However, patients who remain ACL deficient still have atrophy and weakness of the quadriceps for years following injury.

2. To assess inflammatory biomarkers before and after ACL reconstruction to determine if surgery results in a secondary inflammatory response.

   Findings: IL-1β concentrations significantly increased from the day of surgery to the first postoperative time point ($p \leq .001$) and significantly decreased at the 4-week postoperative visit ($p = .03$). IL-1β concentrations at the 4-week postoperative visit remained significantly greater than both

preoperative time points (p >.05). IL-6 concentrations at 1 week post-surgery were significantly higher than at initial presentation (p=0.013), the day of surgery (p<0.001), and 4 weeks after surgery (p=0.002). ACL reconstruction appears to reinitiate an inflammatory response and appears to initiate a second "inflammatory hit".

3. To determine whether muscle-related analytes are differentially expressed in the serum.

   Findings: Proteins chitinase-3-like protein 1 (p= .01), insulin-like growth factor binding protein 1 (p= .01), insulin-like growth factor binding protein 5 (p= .02), renin (p=.004) and lymphotoxin alpha1: beta2 (p= .03) were significantly upregulated in serum following acute ACL injury. The current results confirm the inflammatory pattern previously seen in the synovial fluid thought to play a role in the progression of post traumatic osteoarthritis after ACL injury, and this data also provides further insights into important communication between the joint and quadriceps group, whose function is important in long term health.

4. To use our in vivo pre-clinical model of ACL injury to systematically study the quadriceps muscle at acute time points after injury

   Findings: ACL injury resulted in a decrease in muscle wet weight (p=0.0003) and a trend toward reduced CSA (p=0.06) at 1-week post injury, compared to control. CSA of the ACL injured limb was smaller than that of the contralateral limb at 1-week only (p= 0.01). MAFbx abundance was significantly increased at 48-hours post-ACL injury

(p=0.0001), with no differences for IgG, central nuclei, Pax7, 45s rRNA, total RNA concentration, CD68 or MuRF-1.

Synthesis of Results and Future Research Implications

Several conclusions and implications for future research can be made based on the results of these studies.

1. Patients who remain ACL deficient will have similar long term outcomes to those patients that undergo reconstruction surgery. Although compared to healthy controls and the contralateral side, patients who are ACL deficient will have atrophy and weakness of the quadriceps for years after injury.
2. ACL reconstruction surgery reinitiates the inflammatory response within the knee joint. Furthermore, surgery creates an inflammatory burden that is two-fold higher than the initial ACL injury. The increased inflammatory burden has the potential to influence the systemic and muscular environments, chronic increases in inflammation may contribute to the detrimental side effects of ACL injury like quadriceps atrophy, weakness and the development of OA.
3. Proteomic analysis revealed differences in upregulated proteins between ACL injury and ACL reconstruction related to inflammatory pathways. Additionally, upregulations in muscle related proteins provide a potential connection between the systemic and muscular environments.
4. Pre-clinical models of ACL injury allow for a longitudinal, in depth study of muscle physiology following ACL rupture. We showed that following closed ACL injury there were no indications of inflammation or damage, there was

however, atrophy at one week post injury. Fiber CSA atrophy was preceded by increases in an atrophic marker, this may explain the decrease in muscle size and gives insight into when atrophic processes begin following injury.

Conclusions

This dissertation sought to connect the synovial, serum and muscular environments following ACL injury. We worked to determine how synovial joint inflammation would affect the systemic and muscular environments using both human and animal models. Our data began by elucidating that patients who do not undergo ACL reconstruction surgery and remain ACL deficient will still have atrophy and weakness long term when compared to healthy controls. This indicates that quadriceps atrophy following injury is a result of the ligament rupture and will happen regardless of reconstruction.

Moreover, we found a large increase in synovial fluid inflammatory markers following surgery, almost two fold higher than the injury itself. These data highlight the impact of inflammation on the synovial environment and may have implications for the quadriceps muscle. In response to the large increase in synovial joint inflammation, we found increases in serum circulating inflammatory proteins indicating an increase in inflammatory pathways in the systemic environment. Additionally, there were upregulations in proteins that may be associated in regulating muscle size providing a connection between serum and the quadriceps muscle.

To further characterize the response of the quadriceps we used a novel pre-clinical animal model of closed ACL tear. We looked at time points ranging from hourly after

ACL injury up to four weeks out following injury and found that without the presence of inflammation or damage in the muscle, the quadriceps muscle was still atrophied at one week following injury. This atrophy was preceded by increases in atrophy markers forty eight hours post injury and provides insight into when interventions may be appropriate. These data provide important first steps in characterizing the response of the synovial joint, serum and quadriceps muscle to ACL injury. Longitudinal studies like the one presented here provide valuable insight into when interventions should be applied and potential inflammatory targets. Future studies should focus on continuing to determine the specific communication factors between these environments and developing interventions to combat muscle atrophy following ACL injury.

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PROFESSIONAL PUBLICATIONS

Published Referred Manuscripts

1. **Hunt, E. R**., Baez, S. E., Olson, A. D., Butterfield, T. A., & Dupont-Versteegden, E.E. (2019). Using Massage to Combat Fear-Avoidance and the Pain Tension Cycle. *International Journal of Athletic Therapy and Training*, *24*(5), 198-201.

2. **Hunt, E. R**., Confides, A. L., Abshire, S. M., Dupont-Versteegden, E. E., & Butterfield, T. A. (2019). Massage increases satellite cell number independent of the age-associated alterations in sarcolemma permeability. *Physiological Reports*, *7*(17), e14200.

3. **Hunt, E. R**., Villasanta-Tezanos, A. G., Butterfield, T. A., Lattermann, C., & Jacobs, C. (2020). Upregulation of Systemic Inflammatory Pathways Following Anterior Cruciate Ligament Injury Relates to Both Cartilage and Muscular Changes: A Pilot Study. *Journal of Orthopaedic Research®*, *38*(2), 387-392.

4. Miller, B. F., Hamilton, K. L., Majeed, Z. R., Abshire, S. M., Confides, A. L., Hayek, A. M. **Hunt, E.R.,** ... & Dupont-Versteegden, E. E. (2018). Enhanced skeletal muscle regrowth and remodeling in massaged and contralateral non-massaged hindlimb. *The Journal of Physiology*, *596*(1), 83-103.

5. Van Pelt, D. W., Confides, A. L., Abshire, S. M., **Hunt, E. R**., Dupont-Versteegden, E. E., & Butterfield, T. A. (2019). Age-related responses to a bout of mechanotherapy in skeletal muscle of rats. *Journal of Applied Physiology*, *127*(6), 1782-1791.

6. Jacobs, C. A., **Hunt, E. R.,** Conley, C. E., Johnson, D. L., Stone, A. V., Huebner, J. L., ... & Lattermann, C. (2019). Dysregulated Inflammatory Response Related to Cartilage Degradation after ACL Injury. *Medicine and science in sports and exercise*.

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Accepted Refereed Manuscripts in Press

1. **Hunt, E.R**., Parise, C.N., Butterfield, T.A. The Effectiveness of Non-operative Treatment for Anterior Cruciate Ligament Rupture on Patient Reported Outcomes and Muscular Strength: A Critically Appraised Topic. Accepted 2020. Journal of Sports Rehabilitation.

2. Lepley, L.K., Davi, S.M., **Hunt, E.R**., Burland, J.P., White, M.S., McCormick, G.Y., Butterfield, T.A. Skeletal muscles subjected to eccentrically or concentrically biased exercise exhibit similar morphology with disparate anabolic responses. Accepted, Journal of Athletic Training, 2019.

Manuscripts Submitted for Publication

1. **Hunt, E.R**., Jacobs, C.A., Conley, C.E.-W., Ireland, M.L., Johnson, D.L., Lattermann, C. Anterior Cruciate Ligament Reconstruction Reinitiates an Inflammatory and Chondrodegenerative Process in the knee joint. Submitted, 2019. Journal of Orthopedic Research.