Sex-Specific Anterior Cruciate Ligament Proteomes Following Non-Surgical Rupture

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INTRODUCTION: Over 120,000 anterior cruciate ligament (ACL) injuries occur each year in the United States, accounting for nearly $1 billion in reconstructive surgery costs.[1] Following ACL injury, up to 87% of individuals develop post-traumatic osteoarthritis (PTOA) in their knee joint.[2] PTOA is a debilitating condition that, particularly in its later stages, may require patients to undergo an invasive total knee arthroplasty to restore mobility and alleviate pain.[3] Annual incidence of ACL injuries are increasing and, as a result, PTOA is becoming more prevalent, emphasizing the need for expanded treatment options.[4-7] Since ACL injury is a known risk factor for PTOA, prevention of joint degeneration through enhanced ACL remnant maintenance and reconstruction strategies is a prime candidate for therapeutic intervention. Although it is well established that females are more likely to experience an ACL rupture compared to males, it is poorly understood if sex differences contribute to injury responses of the damaged ligament and development of PTOA.[8-10] Previous studies have demonstrated that male mice selectively minimize voluntary movement and have increased gait modifications relative to female mice following non-surgical (mechanical) ACL injury, potentially indicating sex differences in PTOA development[11]. In this study, we sought to identify differentially abundant proteomic signatures of the ruptured ACL between male and female mice. Establishing early injury responses that may contribute to PTOA progression will assist in the identification of novel strategies for intervention, ultimately improving the lives of both humans and animals that suffer an ACL injury and resultant PTOA development.

METHODS: All animal work was approved by the IACUC at Colorado State University (Protocol #2137). Murine Model of ACL Injury: Unilateral ACL rupture was achieved via mechanical tibial displacement in male and female C57BL/6 mice at 12 weeks of age. At the time of injury induction, mice were anesthetized via 2% isoflurane inhalation and placed in a custom loading system.[12] The ACL loading protocol was then executed: 1) manual preload to 0.7N; 2) preconditioning at 0.4mm displacement for 200 cycles at 1Hz; 3) tibial displacement at 2.0mm displacement (instantaneous loading); and 4) post-rupture tibial displacement test at 0.4mm displacement for 1 cycle at 1Hz. The post-rupture tibial displacement test, as well as post-mortem macroscopic imaging, were used to confirm consistent mid-substance ACL ruptures. Proteomic Sample Processing and Data Analysis: At endpoints of 3-, 7-, and 14-days post injury ACL remnants from ten (10) mice per sex per timepoint were collected under a dissection scope and flash frozen. Intact ACLs from naïve mice were utilized to establish baseline sex differences. Following protein extraction, samples were run at UC Denver Proteomics Core Facility on a Bruker timsTOF SCP Mass Spectrometer for untargeted label-free protein quantification. 2,367 proteins were identified and aligned with Uniprot. Data analysis, statistical comparisons, and pathway analysis were performed with amica v3.0.1[13], Graphpad Prism 9.0.0, and QIAGEN Ingenuity Pathway Analysis respectively. Filtration parameters were set as a minimum of 7/10 valid values in one group required for inclusion. Samples underwent variance stabilization normalization and missing value imputation was performed using a normal distribution downshifted 1.8 standard deviations from the mean with a width of 0.3 standard deviations. Significance for all comparisons was set to adjusted p-values<0.05 and $\log_2$(fold-change) threshold of 1.5. Groups were analyzed using a pair-wise two-way ANOVA for sex and time comparisons and individual proteins were compared using a Student's t-test.

RESULTS SECTION: At baseline, and at all post-injury timepoints, ACLs from female mice demonstrated an elevated proteome response compared to male mice. This variable response between male and female mice peaked at 7 days following injury and gradually resolved by the 14-day time-point. Relative to males, females had baseline increased abundance of immune related proteins such as IGHG2 and IGKC and decreased abundance of the serine protease inhibitor SERPINA1E **(Figure 1)**. With injury, top differentially abundant proteins between males and females included factors associated with extracellular matrix production (COL4A1, COL1A1, SPARC), cell migration/adhesion (POSTN, VTN), and fibrinogen production (FGB, FGA, FGG) **(Figure 2)**.

DISCUSSION: Differentially abundant proteins identified between male and female mice following ACL injury have important roles in ECM production and inflammatory regulation and have been shown to play a role in PTOA development.[14-17] Some of the identified proteins (VTN, SPARC) have been associated with changes to sex hormones, such as estrogen.[18-19] These sex-specific differences suggest that the ACL injury responses leading to PTOA development in males and females could be divergent.

SIGNIFICANCE/CLINICAL RELEVANCE: This study used a mechanical rupture model to investigate how sex differences in the ACL proteome injury response modulate PTOA progression post-injury. The results from this study indicate sex-specific proteome differences of the injured ACL that could perpetuate PTOA development. Future work will examine the downstream effects of modulating sex hormones on these pathways and subsequent PTOA progression. Developing a greater understanding of sex factors on ligamentous injury could aid in the prevention and treatment of PTOA.

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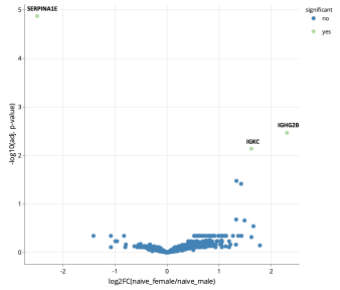

Figure 1. Volcano plot demonstrating significantly differently abundant proteins between naïve male and female mouse ACLs.

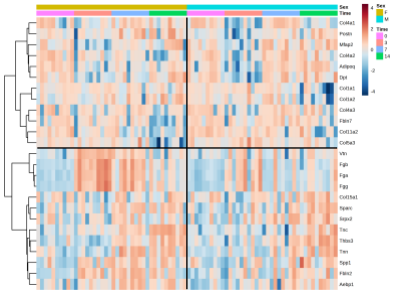

Figure 2. Heat map of proteome clustering demonstrates distinct profiles of ACL proteome variations with both sex and time following injury. Each row represents a distinct protein, and each column denotes an individual mouse sample. The top 25 differentially abundant proteins based on group averages are shown.