Mobility, Metabolic, and Extracellular Matrix Protein Changes Associated with Murine Achilles Tendon Mechanical Overuse

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INTRODUCTION: Tendinopathy is a common and debilitating disease, affecting approximately 25% of all adults [1]. Tendinopathy is characterized by excessive strain and subsequent pathological degeneration to the extracellular matrix of the tendon. Achilles tendinopathy is seen in approximately 6% of the general population [2] and 52% of all runners [3]. Current treatment options for tendinopathy are often either time intensive, costly, or have limited efficacy [4-7]. Therefore, it is crucial to determine novel therapies to mitigate the progression of tendinopathy. Recently, metabolic alterations have been identified in patients with tendinopathy. Patients with systemic metabolic diseases such as diabetes mellitus have increased risk of developing tendinopathy [8]. Furthermore, our lab has shown that tendons exhibit increased metabolism during tendinopathic development Therefore, metabolic targets may modulate disease pathways to mitigate the progression of tendinopathy. The purpose of this study was to challenge murine Achilles tendons *in-vivo* with increasing levels of mechanical overuse and evaluate changes in functional mobility, and tendon extracellular matrix proteins and metabolites.

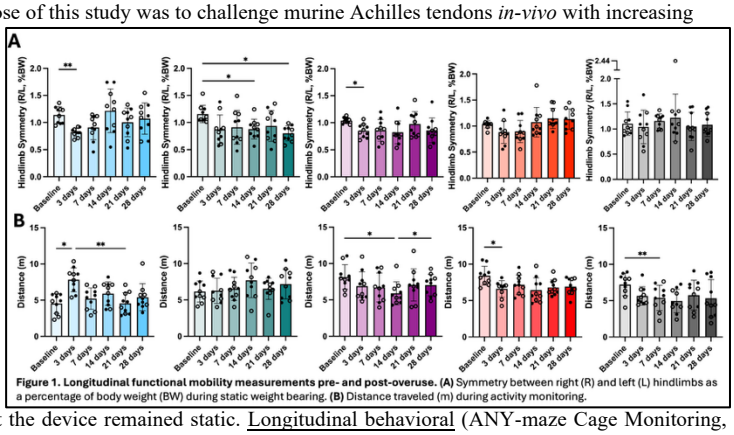

Figure 1. Longitudinal functional mobility measurements pre- and post-overuse. (A) Symmetry between right (R) and left (L) hindlimbs as a percentage of body weight (BW) during static weight bearing. **(B)** Distance traveled (m) during activity monitoring.

METHODS: Animals: 300 (n=150 male, n=150 female) C57BL/6J mice were randomly assigned to thirty experimental groups (n=5 males, n=5 females per group per outcome). Each experimental group was challenged with specific mechanical overuse parameters that mimicked different locomotive gait cycles in mice [9-10]: 1) Slow-Walk (SW); 2) Fast-Walk (FW); 3) Slow-Jog (SJ); 4) Fast-Jog (FJ); 5) Sham (anesthesia control). Groups were either carried out to 14 days or 28 days post-injury. Achilles Overuse Injury Induction: At 15-17 weeks of age, mice were anesthetized, and their right limb was positioned in the custom bioreactor system to allow for ankle flexion/extension with the stifle immobilized. Overuse cyclic loading at a 3.5mm displacement was applied at varying frequencies (SW=2.7Hz, FW=3.4Hz, SJ=4.9Hz, and FJ=5.9Hz) for 30 minutes. The anesthesia control group had their right foot placed in the cup, but the device remained static. Longitudinal behavioral (ANY-maze Cage Monitoring, Stoelting Co.) and static weight-bearing (Incapacitance Test; Bioseb) assessments were conducted at 3-, 7-, 14-, 21-, and 28-days post-injury **(Fig1)**. Animals were acclimated twice with baseline measurements taken in duplicate prior to injury and averaged. Post-mortem outcome analysis: After 14 or 28 days, mice were euthanized and limbs taken for proteomic and metabolomic analysis (data shown), histology (analysis ongoing), and biomechanical testing (analysis ongoing). Untargeted proteomic and metabolomic quantification was performed on each individual tendon using liquid-chromatography mass spectrometry (LC-MS) and analyzed using MetaboAnalyst 6.0 **(Fig2)**. Statistical Analysis: All statistics were conducted in GraphPad Prism 10.1.1 with significance set to $p<0.05$ for all comparisons. Two-way ANOVA with Tukey's post-hoc tests were utilized to compare changes between groups and time.

RESULTS: All mice exhibited anticipated hindlimb symmetry (values close to 1.0) during baseline measurements. Statistically decreased hindlimb symmetry (right vs. left; % of body weight), indicative of injured limb offloading, was observed in the SW group at 3-days post-injury relative to baseline and in the SJ, FW, and FJ groups at 3-, 7-, and 14-days post-injury relative to baseline **(Fig1A)**. Voluntary distance traveled was significantly decreased at post-injury time-points relative to baseline in the FW (7- and 14-days), SJ (3- and 14-days), and FJ (7-days) groups, but not in the SW group **(Fig1B)**. Elevated levels of metabolites at the 14-day timepoint were observed predominantly in the SW and FW groups. Metabolites were also elevated at 28-days for the SW group **(Fig2A)**. Seven (7) of the top fifteen (15) most altered metabolites were amino acids: proline, valine, glutamate, leucine/isoleucine, phenylalanine, alanine, and glutamine **(Fig2B)**. At the 14-day timepoint, arginine, asparagine, aspartate, glycine, histidine, leucine/isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine concentrations were significantly higher compared to the Sham group by two-way ANOVA. At 28-days, only glutamine was significantly lower in the FW group compared to the Sham control (data not shown). Significant direct correlations were seen between collagen type 3 alpha 1 levels and glutamine at 14-days, and lysine, tyrosine, histidine, and leucine at 28-days in the FW group. Significant direct correlations were seen between collagen type 2 alpha 1 levels and glutamine and proline at 28-days (data not shown).

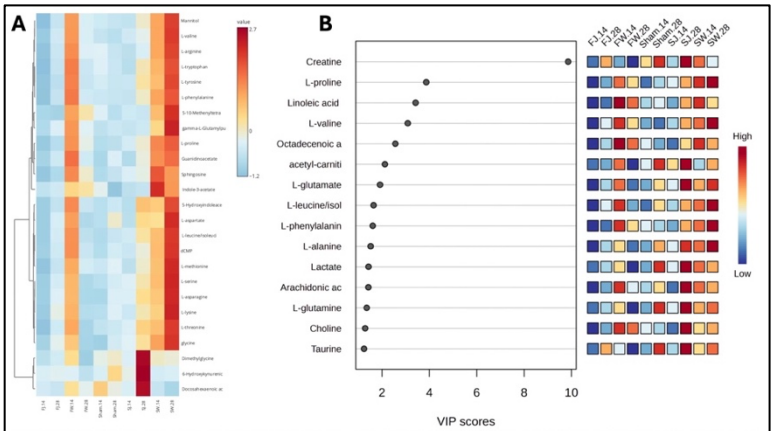

Figure 2. Tendon metabolite concentrations compared across injury and control groups. (A) Heatmap depicting concentrations of metabolites clustered by group, and **(B)** Partial Least Squares Discriminant Analysis (PLS-DA) Variables of Importance (VIP) scores depicting top 15 most altered metabolites based on group.

DISCUSSION: Following Achilles overuse, mice showed changes in functional mobility, with alterations associated with increasing injury severity from slow-walk (SW) to fast-jog (FJ). We hypothesize these acute changes in mobility are associated with Achilles tendon specific degenerative changes, which is being studied with ongoing histologic and biomechanical analysis. Our metabolomic and proteomic results indicate amino acid-associated metabolic pathways are altered following overuse to the Achilles tendon. The upregulation of amino acid metabolites at 14-days, but not 28-days, is indicative of acute metabolic changes which corroborates our previous work.

SIGNIFICANCE: Determining the specific metabolic pathway alterations involved in tendinopathy is a crucial first step to identify and evaluate novel therapeutic targets that modulate these pathways.

REFERENCES: [1] Oreff, et al. Sci Rep (2023); [2] Wang, et al. Sports Med Hlth Sci (2022); [3] Kujala, et al. CJSM (2005); [4] Horterer, et al. AOTS (2023). [5] Kaux, et al, JSM (2011)]; [6] Silbernagel, et al, Br J Sports Med (2007); [7] Silbernagel, et al, AJSM (2007); [8] Nichols, et al. JOR (2019); [9] Hu, et al, Skelet Muscle (2017); [10] Charles, et al, Front. Bioeng. Biotechnol (2018).

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