Potential Sex Differences in the Gut Microbial Response of Mice Following an In Vivo ACL Tear

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INTRODUCTION: Emerging research has established a link between gut microbiome dysbiosis and the development of primary osteoarthritis (OA).[1] However, the association between the gut microbiome and chronic inflammatory conditions (e.g., arthrofibrosis, post-traumatic OA (PTOA)) at high risk of developing following an anterior cruciate ligament (ACL) failure is less understood. Considering the likelihood of developing PTOA is >50% following knee trauma,[2] investigations into potential links between the gut microbiome and post-traumatic disease progression in the knee is warranted. In this pilot study we hypothesized that systemic inflammatory stress triggered by an ACL injury alters the composition of the gut microbiome and qualitatively correlates with localized knee inflammation in both female and male mice.

METHODS: This study was approved by Institutional Animal Care and Use Committee. At 10-weeks-of-age feces were collected from female and male C57BL/6J mice (n=10/sex) under sterile conditions. All mice were then anesthetized, provided heat support, and positioned in a custom loading jig to fail the right ACL in vivo.[3] Mice were then recovered and returned to vivarium. Additional feces were collected from all mice at 1- and 4-weeks post-injury. At each post-injury timepoint, 5 mice per sex were euthanized and their left (control) and right (injured) hindlimbs were removed, fixed, decalcified, and processed for paraffin sectioning. Tissue sections across the medial and lateral knee were stained with safranin-o/fast green for histopathological scoring of articular cartilage degradation and synovitis, and central knee sections were stained for picrosirius red for scoring of arthrofibrosis using previously published methods.[3] Feces collected prior to ACL failure (baseline) and post-injury (1- and 4-weeks) were processed for DNA extraction using a commercial kit specifically optimized for stool samples. Purified DNA was then further prepared for 16S sequencing by a campus genomics core facility. Following quality control measures, taxon mapping, and data normalization, MicrobiomeAnalyst software was used to profile marker gene data across the community and employ shotgun metagenomics to predict microbial function by sex and timepoint.[4]

RESULTS: Histopathology results are provided in Figure 1. Female and male mice at 1-week post-injury demonstrate statistically significant (p<0.01-0.0001) moderate synovial and fibrotic responses in the anterior and posterior knee capsules, but little indication of cartilage degradation. At 4-weeks post-injury all three inflammatory processes are significantly present (p<0.05-0.0001) in injured female and male knees compared to contralateral controls. Importantly, there are no significant differences between males and females in terms of their histopathology scores across post-injury timepoints. When exploring the microbiome of these mice, there were no significant differences in alpha diversity (within-sample diversity) at the feature-level (i.e., smallest captured taxon for each sequence) across timepoints (q=0.70), however there were significant differences between sexes (q=0.0003). Similarly, beta diversity (between-sample diversity) did not differ across timepoints but did between sexes (F=9.5, R^2=0.39, p=0.001). Using linear discriminant analysis (LDA) with effect size (LEfSe), LDA scores were generated to identify significant microbial taxa differences between females and males. Females had 70 amplicon sequence variants that differed from males, while males had 120 that differed from females across timepoints. Predictive functional profiling from the combination of KEGG prokaryotic organisms and normalized taxonomic abundances identified several potential microbial functions that significantly shifted in males between 7- and 28-days post-injury (p<0.05), while females showed no significant microbial function shifts across time (Table 1).

DISCUSSION: Outcomes from this pilot study suggest there are differences in gut microbial communities following an ACL injury between female and male mice, independent of similar degrees of localized knee inflammatory processes. In the acute phase, 7 days post-injury, there appears to be inflammatory dysbiosis in the male microbiome. Functional profiling of taxa suggests metabolic reorganization with the upregulation of amino acid and lipid metabolism and downregulation of DNA repair and carbohydrate metabolism, the latter likely diminishing short fatty chain acid (SCFA) levels and potentially impacting gut barrier integrity.[5] However, females demonstrated little indication of dysbiosis, despite similar degrees of knee inflammation. In the recovery phase, 28 days post-injury, most microbiome functions in males return to pre-injury levels, yet amino acid production remains increased, and cell cycle control, defense mechanisms, DNA repair, and signal transduction remain decreased. This new microbial reality suggests continued microbial stress, amino acid catabolism, and reduced interspecies communication and microbial diversity.[6] Yet again, the female microbiome shows little change.

SIGNIFICANCE/CLINICAL RELEVANCE: With the caveat that this is a pilot in vivo mouse study, the outcomes suggest that in females the gut-joint axis is decoupled following injury, suggesting injury-driven inflammatory response is predominately experienced locally, while the response in males suggests gut-joint connectivity.

REFERENCES: [1]Liu et al., *Front Immunol*, 2023; [2]Lohmander et al., *Am J Sports Med*, 2007; [3]Ahn et al., *Am J Sports Med*, 2023; [4]Chong et al., *Nat Protoc*, 2020; [5]Li et al., *J Hematol Oncol*, 2024; [6]Munley et al., *Microorganisms*, 2023.

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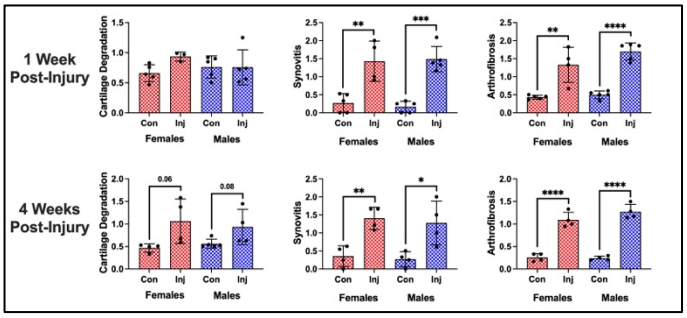

Fig 1. Female and male histopathology scores for cartilage degradation, synovitis, and arthrofibrosis at 1- and 4- weeks post-injury. *p<0.05, **p<0.01, ***p<0.001, p<0.0001. Con=control, Inj=Injured.

Table 1. Female and male functional profiling using KEGG ontology, highlighting functional groups that differ between males and females at 7- and 28-days post-injury. Numbers provided are adjusted p-values and red indicates significance.

	Females		Males			
COG Functional Group	7 days	28 days	7 days	Up/ Down	28 days	Up/ Down
Amino acid transport & metabolism	0.24	0.32	**0.01**	Up	**0.04**	Up
Carbohydrate transport & metabolism	0.21	0.23	**0.03**	Down	0.14	
Cell cycle control, cell division, chromosome partitioning	0.36	0.34	**0.03**	Down	**0.02**	Down
Defense mechanisms	0.20	0.30	**0.00**	Down	**0.01**	Down
Inorganic ion transport & metabolism	0.28	0.36	**0.01**	Up	0.16	
Lipid transport & metabolism	0.25	0.29	**0.01**	Up	0.07	
Replication, recombination, & repair	0.18	0.24	**0.03**	Down	0.06	
Signal transduction mechanisms	0.20	0.30	**0.00**	Down	**0.02**	Down
Transcription	0.19	0.26	**0.04**	Down	0.10	
Translation, ribosomal structure, & biogenesis	0.22	0.31	**0.01**	Up	0.14	