**Metabolic Phenotypes Reflect Patient Sex and Injury Status: A Cross-Sectional Analysis of Human Synovial Fluid**

Running head: Patient sex & injury alter synovial fluid metabolome

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# Abstract

Objective: Osteoarthritis is a heterogeneous disease. The objective was to compare differences in underlying cellular mechanisms and endogenous repair pathways between synovial fluid from male and female participants with different injuries to improve current understanding of the pathophysiology of downstream post-traumatic osteoarthritis.

Design: Synovial fluid from n=33 knee arthroscopy patients between 18 and 70 years with no prior knee injuries was obtained pre-procedure and injury pathology assigned post-procedure. Synovial fluid was extracted and analyzed via liquid chromatography-mass spectrometry metabolomic profiling to examine differences in metabolism between injury pathologies (ligament, meniscal, and combined ligament and meniscal) and patient sex. Samples were pooled and underwent secondary fragmentation to identify metabolites.

Results: Different knee injuries uniquely altered synovial fluid metabolites and downstream pathways including amino acid, lipid, and inflammatory-associated metabolic pathways. Notably, sexual dimorphic metabolic phenotypes were examined between males and females and within injury pathology. Cervonyl carnitine and other identified metabolites differed in concentrations between sexes.

Conclusions: These results suggest that different injuries and patient sex are associated with distinct metabolic phenotypes. Considering these phenotypic associations, a greater understanding of metabolic mechanisms associated with specific injuries, sex, and post-traumatic osteoarthritis development may yield data regarding how endogenous repair pathways differ between male and female injury types. Ongoing metabolomic analysis of synovial fluid in injured male and female patients can be performed to monitor post-traumatic osteoarthritis development and progression.

Keywords: Sex Differences, Injury, Post-Traumatic Osteoarthritis, Synovial Fluid, Metabolomics

# Introduction

Post-traumatic osteoarthritis (PTOA) accounts for approximately 12% of osteoarthritis (OA) cases equating to 5.6 million cases annually1, 2. One of the most prevalent risk factors that contributes to PTOA is joint injury. Within the United States, nearly 250,000 anterior cruciate ligament (ACL) injuries occur annually where approximately 70% of injured patients undergo ACL reconstruction3, 4. ACL injuries are frequently accompanied by damage to other tissues and structures within the knee such as the meniscus5. PTOA prevalence is influenced by the type of injury, where ACL injury alone has a prevalence range between 13-39%, whereas , this range is significantly higher amongst those with combined ligament and meniscal injuries (21-48%)2, 6, 7.

Other patient-specific risk factors that contribute to PTOA include age, body mass index (BMI), and sex. Annually, 15% of knee injuries were attributed to high-school athletes with young female athletes being twice as likely to sustain knee injuries requiring surgical repair compared to male athletes8. Furthermore, females are more likely to develop and experience more severe OA compared to males9, 10. These sex-differences can likely be attributed to hormonal and anatomical differences where females have wider pelvises, small femurs, thinner articular cartilage at the distal femur, a smaller ACL, and a narrower intercondylar notch11, 12.

Although it is established that PTOA is associated with injury as well as other patient-specific risk factors like sex, underlying mechanisms and metabolic alterations following injury at the joint level remain unknown. Examining acute differences in response to various injury types amongst males and females has the potential to positively influence patient treatment, outcomes, and reduce the burden of both PTOA and OA. The application of metabolomic profiling may identify biochemical phenotypes that represent and capture the physiological and metabolic status of the tissue of interest.

A handful of studies have used metabolomic profiling of various samples including blood, urine, and synovial fluid (SF) to understand the pathology of OA13-16. SF is an optimal sample type as it is in direct contact with joint tissue where joint cells (i.e., chondrocytes, bone cells) are secreted into the joint cavity13, 14, 17. Thus, SF content is reflective of joint status during times of health and disease and provides a better representation of joint metabolism compared to blood and urine samples which are diluted in the circulatory compartment. While metabolomics has been applied to SF to underpin OA pathogenesis, the authors are not aware of any study using this method to quantitatively investigate acute metabolic perturbations induced by different knee injuries among males and females. By doing so, the local and systemic response to joint injury can be further examined and has the potential to influence treatment and intervention to benefit overall patient health post-injury in the future.

The primary goal of this study was to compare differences in underlying cellular mechanisms and endogenous repair pathways between synovial fluid from male and female participants with different injuries to improve current understanding of the pathophysiology of downstream post-traumatic osteoarthritis. The secondary goal of this study was to identify differences in pathway regulation and metabolite concentration between male and female participants. To accomplish both goals, metabolites were extracted from injured participants’ SF and analyzed via liquid chromatography-mass spectrometry (LC-MS). Global metabolomic profiling was applied to find specific metabolic perturbations associated with types of injury and patient sex. The identification of dysregulated metabolic pathways and metabolites may underpin mechanisms that differ between injured male and females and may shed light on how PTOA manifest later in life. Moreover, ongoing metabolomic analysis of SF post-repair can be performed in conjunction with measurement of patient outcomes to oversee PTOA development and progression.

# Methods

* 1. *Participant information and inclusion criteria*

In this cross-sectional study, 58 participants were screened for eligibility between July 2021 and February 2022 at Virginia Commonwealth University. Inclusion criteria to participate in this cross-sectional study were (1) age between the age of 18-70 and (2) no history of prior knee injuries, chronic pain, or autoimmune disease(s). Under IRB approval, participant SF was obtained from 45 knee arthroscopy patients prior to repair (Fig, 1, Table 1, Supplementary Table 1). Of the 45 participants, 12 were excluded because the reason for surgery was not related to a traumatic injury (n=11) and inadequate volume of SF (n=1). The time between injury and joint repair was not uniform across all participants, however, varying windows of time (days) between injury and repair did not influence metabolic results. De-identified patient information provided included patient sex, age, BMI, and injury pathology. To limit potential bias, patient information including pathology, BMI, age, and sex were blinded throughout data analysis.

[Suggested location for Figure 1 and Table 1]

* 1. *Synovial fluid sampling, extraction, and metabolic profiling*

For all participants, SF was acquired in the operating room pre-procedure by one of two surgeons, and pathology assignment (i.e., right medial meniscus tear) was assigned post-procedure based on observations made in the operating room and the postoperative pathology report. Participant injury pathologies were categorized into one of three pathology groups: ligament (L), meniscal (M), and combined ligament and meniscal (LM) injuries. A concise overview of the metabolite extraction and MS methods used in this study are discussed in detail in the supplementary material. In brief, all SF samples (n=33) were extracted with methanol and acetonitrile, centrifuged, dried down via vacuum concentration, and prepped for LC-MS analysis. To derive metabolite identifications, two pooled samples containing extracted SF from samples selected at random were prepped for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis which entails fragmentation of parent ions. All sample data, including chromatograms and spectra, were manually inspected to determine if any issues arose during the mass spectrometry run. In doing so, two samples (n=2) were removed from the analysis as they resembled quality control blanks and high levels of background noise.

All data (n=31) were processed using MSConvert18 and XCMS19. MetaboAnalyst20 was utilized to statistically analyze samples, visualize dissimilarities, and pinpoint pathway dysregulation between males and females with different injuries. To identify metabolite features data from LC-MS/MS data of pooled samples were analyzed with Progenesis QI (Nonlinear Dynamics, Newcastle, UK). Full details on metabolomic profiling, including statistical and pathway analyses, and metabolite identification can be found in the supplementary material.

# Results

* 1. *The synovial fluid metabolome differs by injury pathology*

In total, 7,794 metabolite features were detected by LC-MS in the 33 SF samples. To assess global differences between injury pathologies, all metabolite features were analyzed using Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) (Fig. 2A-B). PCA displayed overlap of groups with PC1 and PC2 accounting for 40.1% of the variability in the dataset, whereas PLS-DA showed less overlap with Components 1 and 2 accounting for 31.6% of the variability in the dataset.

[Suggested location for Figure 2]

Although distinct metabolic patterns at the global level showed overlapping groups, the top 25 highest PLS-DA Variable Importance in Projection (VIP) scoring metabolites had scores > 2.2 and were selected for further analysis to examine and pinpoint phenotypic differences in regulation between pathology groups. VIP scores reflect how much a variable contributes to the model and are calculated by summing the squared correlations between the PLS-DA components and the original value with appropriate weighting. Of the 25 metabolite features, 8 were the most abundant in M injuries, the least abundant in L injuries, and intermediary abundance levels in LM injuries (Fig. 3A Cluster 1). Conversely, 8 of the 25 metabolite features were the most abundant in L injuries, the least abundant in M injuries, and intermediary abundance levels in LM injuries (Fig. 3A Cluster 2). Lastly, 9 metabolite features were similar in abundance in L and LM injuries, and lowest in M injuries (Fig. 3A Cluster 3). Additionally, PLS-DA VIP metabolites features with a score < 2 were then matched to identifications made using LC-MS/MS. In total, 7 metabolites were identified where tryptophanoland gandoeric acid were highest in abundance among LM participants, whereas isoleucyl-proline, alpha linoleic acid, linoleic acid, lansiumamide C, and eriojaposide B were highest in abundance among M injuries (Supplementary Table 2).

[Suggested location for Figure 3]

Functional pathway enrichment analyses were conducted to underpin endogenous repair pathways that differ between injury pathologies. To do so, a median metabolite intensity heatmap analysis was performed to visualize global changes across the metabolome to distinguish patterns, or clusters, of co-regulated and differentially expressed metabolite features (Fig. 3B). Clusters of metabolites identified underwent pathway enrichment analyses using MetaboAnalyst’s functional analysis feature. Conversely, features that had the highest concentration in M injuries, and lowest in L injuries, mapped to lipid-related pathways (fatty acid oxidation and activation, glycosphingolipid metabolism, omega-6 fatty acid metabolism), histidine metabolism, TCA cycle, and glycolysis (Fig. 3B Cluster 1). Arginine and proline metabolism was detected in both L and M injuries and was lowest in LM injuries (Fig. 3B Clusters 1&2). Additionally, those highest in M injuries but lowest in LM injuries mapped to other lipid-related pathways (mono-unsaturated fatty acid beta-oxidation, omega-3 fatty acid metabolism, fatty acid biosynthesis, saturated fatty acids beta-oxidation, dimethyl-branched-chain fatty acid mitochondrial beta-oxidation) (Fig. 3B Cluster 5). Metabolite features that had the highest concentration in LM injuries, and the lowest in L injuries, mapped to amino acid metabolism (lysine, glycine, serine, alanine, threonine), trihydroxycoprostanoyl-CoA beta-oxidation, proteoglycan biosynthesis, and vitamin B3 metabolism (Fig. 3B Cluster 2). Additionally, features highest in LM injuries, but lowest in M injuries, mapped to phosphatidylinositol phosphate metabolism, the carnitine shuttle, and linoleate metabolism (Fig. 3B Cluster 6). Metabolite features that had the highest concentration in L injuries, and lowest in M injuries, mapped to sialic acid metabolism, aspartate and asparagine metabolism, and glycerophospholipid metabolism (Fig. 3B Cluster 7). Additionally, biopterin metabolism was highest in L injuries but lowest in LM injuries (Fig. 3B Cluster 3) (Table 2, Supplementary Table 3). All pathways reported had an FDR-corrected p-value < 0.05.Taken together, the regulation of each pathology group is distinct further supporting the notion that the SF metabolome differ by injury pathology. However, we are unable to speculate as to why key metabolites from each of the L and M groups is not necessarily as prominent in the LM group.

[Suggested location for Table 2]

* 1. *Participant sex influences metabolomic profiles across injury pathologies*

To determine if participant sex influences the SF metabolome, PCA, PLS-DA, and volcano plot analyses were conducted. PCA displayed overlap (Fig. 4A) whereas PLS-DA revealed some overlap of male and female participants suggesting the SF metabolome reflects participant sex (Fig. 4B). While overlap was observed in both PCA and PLS-DA, this can possibly be attributed to analyzing all participant SF considering only the factor of sex. To further investigate potential sexual dimorphism, volcano plot assessed male and female participant SF-derived metabolites using significance and fold change. This analysis identified 6 metabolites features that were significant and had higher concentrations in males compared to females, and 35 metabolite features that were significant and in higher concentration in females compared to males (FC > 2, p < 0.05) (Fig. 4C).

[Suggested location for Figure 3]

In a similar way, pairwise comparisons were performed to examine participant sex differences within injury pathology for L and M injuries. LM injuries were not examined due to insufficient sample size (LM Male n = 4, LM Female n = 1). Considering L injuries, PCA displayed some overlap (Fig. 4D) while PLS-DA displayed clear separation of participants that differ by sex (Fig. 4E). Volcano plot analysis identified 55 metabolite features that were significant and had higher concentrations in L males, whereas 38 were significant and in higher concentrations in L females (FC > 2, p < 0.05) (Fig. 4F).

These same analyses were performed to analyze metabolic phenotypes associated with male and female M injuries. PCA displayed some overlap (Fig. 4G), whereas PLS-DA showed near complete separation of male and female participants with M injuries, further supporting the notion that differences at the metabolic differences are associated with participant sex (Fig. 4H). Volcano plot analysis identified 89 metabolite features that were significant and had higher concentration in M males, and 105 features that were significant and had higher concentrations in M females (FC > 2, p < 0.05) (Fig. 4I).

Populations of metabolite features from volcano plot analyses were then matched to identifications made using LC-MS/MS data. As expected with LC-MS/MS, not all metabolite features were able to be identified (Supplementary Table 4). Of the 18 identified metabolites, a noteworthy metabolite that was statistically significant across all volcano plot analyses was cervonyl carnitine. Specifically, cervonyl carnitine was detected in higher abundances in all females compared to males and when considering injury pathology) (Fig. 5A-C). Although an identified metabolite was not higher in abundance in all males compared to males and when considering injury pathology, identified metabolites that were higher in abundance in males compared to females included alpha-Chaconine (M males), Lucidenic acid A (M males), lysine (L males), arginine (M males), as well as others (Supplementary Table 4). Overall, the detection of distinct global phenotypes and differences in regulation of identified metabolites between male and female participants strongly suggests that participant sex influences SF metabolism across injury pathologies.

[Suggested location for Figure 4]

# Discussion

To our knowledge, this is the first study to examine acute metabolic responses following different injury types in male and female human SF using mass-spectrometry. The goals of this study were to (1) identify metabolic perturbations that differ across injury pathologies including L, M, and combined LM injuries and (2) examine metabolic differences associated with sex. By applying mass spectrometry-based metabolomics, metabolic phenotypes between injury pathologies vary suggesting different types of injury trigger specific acute metabolic responses. Additionally, SF-derived metabolites reflected participant sex, and male and female participants with similar injuries were metabolically distinct from each other. The detection of metabolites and generation of metabolomic phenotypes based on sex and injury pathology can be used to improve patient treatment and allow for more precise treatment to benefit joint and patient health post-injury.

* 1. *Acute metabolic responses post-injury varies based on injury pathology*

Previous studies used metabolomics to examine post-injury metabolic shifts in animal models17, 21, 22, and only one study to date has examined metabolic shifts in human SF induced by different injuries using nuclear magnetic resonance spectroscopy (NMR)23. This study detected significant changes in glucose, amino acids, lipids, and lactate indicating increased demand for these compounds within the joint post-injury. The results of this cross-sectional study closely align with previously generated metabolic phenotypes, showing similar pathway regulation differences between injuries. Specifically, differences in lipid- and oxidative-related metabolism (M injuries), amino acid metabolism (all injuries), and inflammatory-associated pathways (LM injuries) differed in regulation between injury pathologies (Fig. 6).

[Suggested location for Figure 6]

Participants with L injuries showed the greatest dysregulation of sialic acid metabolism compared to other injuries. Sialic acid plays a vital role in cell differentiation, proliferation, and inflammation. Previous studies have linked elevated plasma sialic acid concentration to inflammation24, 25 and OA26. Enriched sialic acid metabolism in SF from L participants may reflect joint inflammation post-injury and could be monitored long-term. However, additional investigation is needed to understand the relationship between sialic acid and joint inflammation following injury and downstream PTOA development.

Aspartate and asparagine metabolism was the most dysregulated in participants with L injuries compared to other injuries. Both amino acids have anti-inflammatory effects and are found in low concentrations in OA patient serum27, 28. Sheep SF analysis post-ACL injury also showed increased levels of these amino acids compared to non-injured controls17. Proline and arginine metabolism was detected in both L and M injuries and was the lowest in LM injuries. Proline, a downstream product of arginine, contributes to collagen synthesis29 and has been associated with OA28. A prior study detected higher concentrations of these amino acids in SF immediately following injury that then decreased overtime in a non-invasive mouse ACL tear model22 leading authors to hypothesize that these amnio acids could be markers of injury but further investigation is needed.

Amongst M participants, lipid-related oxidative metabolic pathways were the most dysregulated compared to L and LM participants. During normal conditions, SF relies on lipid species to provide lubrication and reduce joint friction, therefore, this dysregulation likely reflects the lubrication and inflammatory statuses of the joint tissue post-M injury. Moreover, the dysregulation of fatty acid biosynthesis, oxidation, and degradation as well as glycolysis and the TCA cycle across participants with M injuries may reflect mitochondrial health and dysfunction in other joint tissues post-injury. Functioning mitochondria rely on glucose and fatty acids to yield energy, but dysfunctional mitochondria switch to relying on fatty acids more than glucose30, 31. This can lead to the accumulation of fatty acids32, 33 resulting in lower ATP production, impaired stress response, an increase in reactive oxygen species, apoptosis, and combined can lead to systemic irreparable damage34. Previous studies have also found dysregulated lipid metabolism in SF from OA patients13 and altered fatty acid metabolism in injured mice21, suggesting its significance in joint health.

Previous metabolomics studies on SF and the results of this cross-sectional study suggest that M injuries may trigger mitochondrial dysfunction leading to an influx of fatty acids into the mitochondria where they become the primary energy source. This could cause accumulation and potential development of PTOA, or early-stage OA develop. The response appears to be more pronounced in M injuries, indicating distinct metabolic mechanisms are associated with specific injuries, Further investigation into metabolic regulation following injury could provide insights into the contribution of endogenous repair pathways to PTOA development.

Linoleate metabolism was the most dysregulated pathway among LM participants compared to L and M participants. Linoleic acid, a prominent fatty acid in SF, may be related to the degree of joint disease based on previous studies35, 36. This pathway is upstream of arachidonic acid metabolism which generates fast-acting and short-lived signaling molecules such as the pro-inflammatory prostaglandin, PGH2, by cleaving and converting arachidonic acid37, 38. Arachidonic acid has been detected in OA SF, with higher levels in early stages and lower levels in late-stage OA38, 39. Furthermore, arachidonic acid metabolism was upregulated in SF from both naïve and germ-free mice following non-invasive ACL injury compared to healthy non-injured mice21. Therefore, the detection of linoleic acid and metabolites in this study may be associated with signaling, prostaglandin generation, and inflammation post-LM injury. Larger studies with SF samples from healthy and PTOA individuals are needed to confirm if metabolites related to linoleate metabolism could serve as markers to monitor PTOA development.

Combined, the magnified dysregulation of amino acid-related pathways in LM participants, and fewer in M and L participants, suggest that traumatic joint injuries in general require a higher cellular demand for amino acids for various reasons (i.e., energy generation, collagen synthesis, inflammation). Few studies have determined the cellular density in healthy and injured menisci and ligaments40-42, making it valuable to combine cellular density data with metabolomics for better insights into amino acid metabolism post-injury. Additional studies comparing the SF metabolome from healthy non-injured, recently injured, and PTOA patients are needed to fully comprehend the metabolic mechanisms driving PTOA progression.

* 1. *The synovial fluid metabolome differs in association with participant sex and injury pathology*

Females have a higher risk of knee injury and PTOA development compared to males8-10. Despite empirical sex differences, diagnosis and treatment do not differ43. Therefore, the detection of sex differences at the metabolic level in the present study support sex as a prevalent PTOA risk factor. SF-derived metabolite features were associated with participant sex, and metabolically distinct profiles were observed between males and females with similar injuries. This is the first study, to our knowledge, to assess sex differences considering different injury pathologies in human SF.

Cervonyl carnitine was higher in all female participants compared to all male participants. Carnitines regulate oxidative and metabolic statuses, maintain membrane stability, and contribute to β-oxidation by transporting fatty acids into mitochondria44, 45. Circulating estrogen levels are much higher in females than males, resulting in an increased expression of fatty acid oxidation proteins and pathways such as adenosine monophosphate-activated protein kinase (AMPK)46, 47. AMPK is a key energy sensor that promotes ATP production48, 49. The proposed mechanism for estrogen activation of AMPK entails estrogen binding to estrogen-receptor β (ERβ), causing an increase in Ca2+ stimulating Ca2+/Calmodulin-dependent protein kinase kinase β (CaMKKβ) to phosphorylate the AMPKα subunit where anabolism is inhibited and catabolism is activated resulting in the transport of fatty acids into the mitochondria to perform β-oxidation and generate ATP50 (Supplementary Fig. 1).

Atypical AMPK activity has been implicated in OA where impaired mitochondrial function increases reactive oxygen species and decreases ATP production causing cartilage degeneration, inflammation, and abnormal subchondral bone remodeling49. Therefore, elevated carnitine species in females compared to males may demonstrate that females rely on different metabolic pools and mechanisms, like AMPK activation via estrogen, to meet energy demands post-injury. Monitoring carnitine-related species overtime could gauge β-oxidation rates, AMPK activity and function, joint health, and predict PTOA onset and development. Additional research is needed to explore the sexual dimorphic nature of traumatic knee injury in both human and animal injury models and enhance current understanding of the relationship between injury and sex. These findings underscore patient sex as a significant risk factor for injury and PTOA, warranting further investigation to positively influence patient treatment strategies.

* 1. *Limitations*

While this study finds that metabolic phenotypes reflect patient sex and injury pathology, it is not without limitations. Firstly, a major limitation is the lack of healthy non-injured participants as well as participates with PTOA which limits analyses and study conclusions. Secondly, sample size in general was small and was not uniform across injury pathology groups. Thirdly, a common limitation of LC-MS/MS is that not all metabolite features detected are able to be identified. Therefore, metabolite features that best reflect injury pathology may not have been identified in the current study but have noteworthy metabolic regulation patterns that can be further pursued in additional studies.

* 1. *Conclusions*

The results of this cross-sectional study demonstrate that injured males and females have distinct metabolic phenotypes. Considering sex differences associated with injury rates and PTOA development, the detection of dysregulated metabolites between male and females can be used to positively influence patient treatment and intervention. Future studies aim to investigate the interaction between PTOA, sex, and other PTOA risk factors (i.e., BMI, age). This mass spectrometry-based global approach differentiated participants with L, M, and LM injuries highlighting differences in lipid- and oxidative-related metabolism, amino acid metabolism, and inflammatory-associated pathways. Additional comparison of these metabolic phenotypes to healthy non-injured and PTOA phenotypes is needed to pinpoint joint-level metabolic activity post-injury over time. Completion of this work has the potential to improve patient treatment and identify biomarkers and druggable targets to slow, stop or reverse PTOA progression.

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**Author Contributions**

HDW designed experiments, performed metabolite extractions, ran LC-MS samples, analyzed and interpreted data, and drafted manuscript. AHW performed metabolite extractions and analyzed and interpreted data. PP, JS, RO, and ARV obtained samples and provided clinical input. AV and RO performed repairs and assisted in conception and designed experiments. BB and RKJ designed experiments, analyzed data, drafted and edited the manuscript. All authors have read and revised the manuscript.

**Conflicts of Interest**

Authors have no conflicts of interest to disclose. Dr. June owns stock in Beartooth Biotech and OpenBioWorks which were not involved in this study.

**Abbreviations List**

PTOA = post-traumatic osteoarthritis

OA = osteoarthritis

ACL = anterior cruciate ligament

BMI = body mass index

SF = synovial fluid

LC-MS = liquid chromatography-mass spectrometry

LC-MS/MS = liquid chromatography tandem mass spectrometry

PCA = principal component analysis

PLS-DA = partial least squares-discriminant analysis

VIP = variable importance in projection

NMR = nuclear magnetic resonance

AMPK = adenosine monophosphate-activated protein kinase

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# Figures and Tables

**Flow-flow diagram of the study

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**Figure 1. Participant eligibility and screening.**

A comparison of a diagram

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**Figure 2**. **Global profiles considering all detected metabolite features show moderate differences between injury pathologies.** (A) Principal component analysis, an unsupervised test, shows some overlap when considering all 7,794 features and accounts for 40.1% of the variability in the dataset. (B) Partial least squares-discriminant analysis, a supervised test, similarly shows some overlap of groups and accounts for 31.7% of the variability in the dataset. These two tests combined suggest that global profiles generated by all metabolite features detected somewhat differ and that additional analyses are required to pinpoint specific phenotypic changes. The colors in A and B correspond to: Ligament injuries - light blue; Meniscal injuries - red; Ligament and Meniscal injuries - yellow. L = ligament injuries. M = meniscal injuries. LM = ligament and meniscal injuries.

**A close-up of a chart

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**Figure 3. Heatmap analyses of participants reveal metabolite features and metabolic pathways that are differently regulated between injury pathologies.** (A) Hierarchical clustering analysis visualized by a group median heatmap of the 25 PLS-DA Variable in Importance Projection Scores shows that different injury pathologies trigger distinct metabolic regulation patterns post-injury. Clusters 1-3 (C1-C3) highlight different regulation patterns between injuries. (B) Clusters of coregulated metabolite features within synovial fluid from participants with different knee injuries indicate that distinct metabolic phenotypes across injury pathologies. Clusters (1-3) were subjected to pathway enrichment analyses to identify pathways that differed in regulation between injury pathologies. Warmer colors (yellow) and cooler colors (blue) indicate higher and lower metabolite intensities, respectively. The colors in A-C correspond to: Ligament injuries - light blue; Meniscal injuries - red; Ligament and Meniscal injuries - yellow. L = ligament injuries. M = meniscal injuries. LM = ligament and meniscal injuries.

**A collage of graphs showing different types of circles

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**Figure 4.** **Metabolomic profiles of synovial fluid-derived metabolite features are associated with participant sex and injury pathology.** (A) PCA and (B) PLS-DA display some overlap when comparing all male and female participant suggesting that the SF metabolome is potentially influenced by participant sex. (C) Volcano plot analysis identified 35 etabolite features in females that had a FC > 2 and p-value < 0.05. Conversely, 6 had a FC < -2 and a p-value < 0.05 in males. Similarly, (D) PCA, (E) PLS-DA, and (F) volcano plot analysis was applied to examine metabolic differences between male and female participants with ligament injuries and the same suite of analyses were applied to identify differences between male and female participants with meniscal injuries (G-I). Considering the identification of subpopulations of metabolite features that differ in regulation between male and female participants (A-C), male and female participants with ligament injuries (D-F), and male and female participants with meniscal injuries (G-I), it is evident that the metabolome is influenced by participant sex and injury pathology. The colors in A-I correspond to male and female participants: pink – females, blue – males. L = ligament injuries. M = meniscal injuries.

A diagram of two people

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**Figure 5. Cervonyl Carnitine differs in concentration between male and female participants.** The identified metabolite, Cervonyl carnitine, was higher in concentration in (A) all female participants compared to males, (B) in females with ligament injuries and (C) meniscal injuries compared to males within the same injury pathology group. Mass-to-charge intensities of interest were normalized and used to generate plots. To correct for multiple comparisons, FDR p-value corrections were performed and were less than < 0.05. The colors correspond to male and female participants: pink – females, blue – males.

A diagram of a cell cycle

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**Figure 6. Alterations in synovial fluid metabolism post-injury are associated with different injury types.** Metabolites that differ in abundance across ligament, meniscal, and ligament and meniscal injuries are associated with amino acid, lipid, and inflammation-related pathways. Multiple amino acids were detected within each injury pathology. Amino acids bolded in blue, red, and yellow were detected in synovial fluid from participants with ligament, meniscus, and ligament and meniscus injuries, respectively. Metabolite features detected among participants with meniscal injuries mapped to lipid-related pathways including fatty acid activation, oxidation, and biosynthesis, beta-oxidation related pathways, and others (red). Ligament and meniscal injuries were associated with linoleate metabolism, which is related to arachidonic acid metabolism and the generation of prostaglandins and eicosanoids (yellow). Additionally, the metabolite features involved in the carnitine shuttle was detected in high abundances in participants with ligament and meniscal injuries. L = ligament injuries. M = meniscal injuries. LM = ligament and meniscal injuries.

**Table 1.** Participant information. Values indicate number of participants in each group.



**Table 2.** Metabolic pathways associated with ligament, meniscal, and ligament and meniscal injuries identified by median metabolite intensity heatmap analysis. All reported pathways have a FDR-corrected significance level < 0.05.L = ligament injuries. M = meniscal injuries. LM = ligament and meniscal injuries. Clusters defined in Figure 3B.

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# Supporting Information

*Note that there is an additional file with supplementary methods, as well as additional files for supplemental tables.*

**Diagram of a diagram of a cell block

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**Supplementary Figure 1. Proposed mechanism of AMPK activation by estrogenSupplementary Table Captions**

**Supplementary Table 1.** Participant information for all participants including BMI, age, sex, time between injury and repair (days), reason for knee arthroscopy repair, and injury pathology assignment.

**Supplementary Table 2.** Identified putative metabolites that differ in abundance between injury pathology and have a variable importance in projection score greater than 2 identified by liquid chromatography tandem mass spectrometry (LC-MS/MS). For all identified putative metabolites provided information includes observed mass-to-charge ratios, theoretical mass-to-charge ratios from Progenesis, ppm error, accepted human metabolome database compound identification number, accepted description, adduct, chemical formula, total score, and fragmentation score. Identifications with error > 20 ppm, overall score < 60, and fragmentation score < 12 were excluded.

**Supplementary Table 3.** All metabolic pathways determined from MetaboAnalyst when comparing participants with different injury pathologies (Ligament, Meniscal, Ligament and Meniscal injuries) using median metabolite intensity heatmap analysis. Clusters defined on Figure 3B. No statistically significant pathways were detected in cluster 4.

**Supplementary Table 4.** Identified putative metabolites that differ in abundance between male and female participants identified by liquid chromatography tandem mass spectrometry (LC-MS/MS). For all identified putative metabolites provided information includes observed mass-to-charge ratios, theoretical mass-to-charge ratios from Progenesis, ppm error, accepted human metabolome database compound identification number, accepted description, adduct, chemical formula, total score, and fragmentation score. Identifications with error > 20 ppm, overall score < 60, and fragmentation score < 12 were excluded.