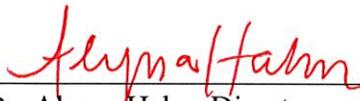
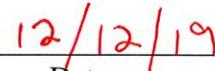


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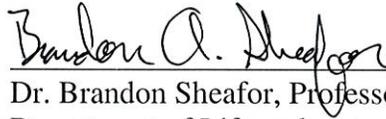
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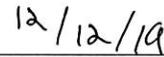
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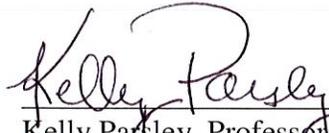
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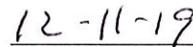
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Date

Global metabolic profiling of human joint fluid following a second ACL tear: A case
report

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Abstract

Objectives: The objective of this study was to investigate the acute metabolic response to injury using global, or untargeted, metabolic profiling. The purpose was to better understand the acute response to injury following a second traumatic joint injury.

Methods: This study is a case report of a patient who had sustained two separate ACL tears in the same knee years apart. We extracted metabolites from healthy synovial fluid and human joint fluid following the patient's second ACL tear and analyzed the metabolite extracts using liquid chromatography-mass spectrometry (LC-MS)-based global metabolic profiling.

Results: We identified 1241 unique metabolite features in injured SF compared to healthy SF (Student's t-test: FDR-adjusted p-value <0.05). Principal component analysis (PCA) and hierarchical cluster analysis (HCA) further confirmed distinct, unsupervised separation between experimental groups. We also identified 11 potential discriminatory metabolites following a second ACL tear, 4 enriched pathways through EBAM (FDR-corrected p-value < 0.05) (urea cycle/amino group metabolism; arginine and proline metabolism; glycerophospholipid metabolism; and lysine metabolism), and 23 additional enriched pathways through PLS-DA VIP scores.

Conclusion: These results potentially provide insight into the biochemical response immediately following a second ACL tear in a single patient. This insight could be useful for targeting specific pathways to prevent or delay PTOA.

Introduction

Posttraumatic Osteoarthritis (PTOA) is a subtype of osteoarthritis (OA) caused by acute injury to the joint and can occur both with disruption of the joint intraarticular surface (intraarticular fractures and cartilage damage) or without disruption of the intraarticular surface (meniscal, ligament, and joint capsule tears) (1-9). It is characterized by swelling, synovial effusion, and joint degeneration. Patients often present with pain, joint stiffness, and disability (8). While OA typically affects adults older than 60 years, PTOA is more common in adults younger than 60 years old who have sustained a joint injury (8-13).

Although the pathophysiology of PTOA is not well understood, individuals with a significant ligamentous or capsular injury are up to 10 times more likely to develop OA, and those with an intraarticular fracture are up to 20 times more likely (13-17). PTOA is most common in the ankle (54-78%) followed by the knee (12.5%), and although less common, can also occur in the shoulder and hip (8, 18-21). Anderson et al. (2011) found that the presence of PTOA correlates with both the impact energy and degree of articular surface damage caused by the injury (13). A number of other risk factors can contribute to PTOA including joint instability, differences among joints, patient age, genetics, physical activity, and patient sex (1, 9, 22, 23). PTOA generally is not diagnosed until the onset of symptoms, which often happens after irreversible damage has occurred in the joint (8). Therefore, more information is needed to clinically diagnose PTOA earlier and to intervene sooner in order to potentially prevent further damage.

Current research suggests that acute joint injuries initiate biochemical responses that lead to degradation of the joint and to the development of PTOA (13). A systematic

review of the pathogenesis of PTOA confirmed that an inflammatory response occurs shortly after joint injury and is sustained at low levels over time (24). Studies suggest that this acute response may mark the beginning of OA pathogenesis (24). While many past studies have focused on the acute inflammatory response, no study to date has investigated global changes in metabolism in the acute phase following joint trauma. Importantly, patients typically seek medical attention during this acute phase, presenting an opportunity for intervention. Thus, a greater understanding of this acute response to injury and its implications for PTOA pathogenesis could lead to potential drug targets to prevent the onset of disease

Anterior cruciate ligament (ACL) injuries are among the most common types of knee injuries and often occur with damage to the cartilage, subchondral bone, collateral ligaments, and menisci (9). It is estimated that half of all patients with an ACL tear will develop PTOA in the knee (25). Additionally, after one ACL tear, the risk of a second ACL tear is 20-30% (26-30). The most common treatment for an ACL tear is reconstruction surgery with a mean lifetime cost of \$38,212 (31). It is unclear if surgery decreases the likelihood of PTOA by better stabilizing the joint, or increases the likelihood by traumatizing the joint a second time (31). Therefore, it is important to better understand the pathophysiology of PTOA following an ACL tear to determine the most effective treatment and identify prevention strategies for early intervention.

There are a variety of joint tissues that could be investigated to better understand OA. In OA, all of the surrounding tissues are involved in the degradation of the joint, including the articular cartilage, subchondral bone, joint capsule, synovium, and synovial fluid (SF) (32, 33). Of all of these, SF is a promising tissue to examine for gaining

understanding of OA. Synovial fluid is produced by synovium and functions to reduce friction that is generated through joint movement. It is an ideal snapshot of the metabolome of the joint because it is in immediate contact with the other diseased tissues, so metabolites from other parts of the joint can be detected in SF (34). Therefore, metabolites in SF may provide insight into the development of PTOA, identify potential biomarkers predictive of PTOA, and present targets for PTOA prevention and treatment.

To study the condition and present state of the joint, metabolomics, which reflects real-time changes in the metabolome through identification of metabolites, can be used. While mass spectrometry (MS) and nuclear magnetic resonance (NMR) are both techniques used for metabolomics, MS is more specific and sensitive (35). Further, liquid chromatography paired with MS offers more accurate analysis because it better handles complex mixtures (36). Consequently, LC-MS is a common and well-accepted technique for analysis of metabolomics.

The objective of this study was to investigate the acute metabolic response to injury using global, or untargeted, metabolic profiling. To accomplish this, we extracted metabolites from healthy synovial fluid postmortem as well as human joint fluid following a traumatic joint injury and analyzed the metabolite extracts using liquid chromatography-mass spectrometry (LC-MS)-based global metabolic profiling.

The human joint tissue obtained following traumatic injury was collected from a patient who had sustained two separate ACL tears in the same knee years apart. Due to the trauma of two injuries, this patient not only is very likely to develop PTOA, but may also have an altered metabolome resulting from the first ACL tear which may be predictive of early PTOA. Thus, we hypothesize that the joint fluid would have much

higher levels of inflammatory markers and potentially similar markers for OA due to the possibility of PTOA acquired from the initial ACL tear.

Methods

Double ACL tear joint drain fluid and synovial fluid samples

Joint drain fluid (n = 1) was obtained under IRB approval from a patient who sustained two distinct ACL tears in the same knee years apart. The joint fluid was gathered by the patient's physician from an intra-articular drain placed post-operatively following ACL reconstruction. Due to the statistical problems associated with a single sample, five samples were taken from the patient's drain fluid. Healthy SF samples used for comparison were purchased from Articular Engineering (Northbrook, IL). These samples were collected post mortem and partial clinical data including age, gender, race, and cause of death were supplied. The samples were frozen at -80°C after harvest until analysis.

Metabolite extraction and mass spectrometry

Metabolite extraction was performed using techniques similar to those described by Carlson et al (2018) (34). Prior to analysis by LC-MS, metabolites were extracted from 100 µL of joint fluid. Samples were thawed on ice for 3-5 minutes and then centrifuged at 500 x g for 10 minutes at 4°C to remove excess cells and debris. The supernatant was collected, evaporated, and re-suspended in 100 µL of 50:50 water:acetonitrile. 500 µL of acetone was added to the sample before it was placed on a shaker for 4 minutes. This process helped to precipitate the polymers before 10 minutes of refrigeration. The samples were then centrifuged at 16100 x g for 5 minutes at which time the supernatant was removed and further evaporated in a new tube. The remaining

sample volume was doubled with acetonitrile making a final 50:50 water:acetonitrile LC-MS injection buffer. An Agilent 1290 UPLC with a Cogent Diamond Hydride HILIC 150 x 2.1 mm column (MicroSolv, Eatontown, NJ) and Agilent 6538 Q-TOF mass spectrometer (Agilent Santa Clara, CA) were used to analyze mass spectrometry-prepared metabolite extracts using a normal phase gradient elution method and a positive mode.

Statistical Analysis

Global metabolomics creates a large dataset consisting of thousands of mass-to-charge ratios and their peak intensities. The data set was decreased by removing metabolite features with median intensity values of zero. Statistical analyses were performed on the remaining data using MetaboAnalyst (Xia Lab, McGill University, Quebec CAN). The data was log transformed (\log_2), normalized, and auto scaled (mean-centered and divided by the standard deviation of each variable).

Multivariate analyses assessed overall differences between healthy and injured joint fluid. Unsupervised hierarchical clustering analysis (HCA) grouped distinct subsets of samples together and was visualized using a dendrogram. Principal component analysis (PCA) further distinguished unsupervised differences between samples and was mapped with a 2D scores plot.

Specific differences between groups were identified using five methods. 1) Student's t-tests showed which metabolite features were significantly different, and 2) volcano plot analyses showed which metabolite features were significantly upregulated or downregulated. 3) Partial least squares-discriminant analyses (PLS-DA) were used to identify which metabolite features contributed the most to distinction between known

groups from PLS-DA. 4) Significance Analysis of Microarray (and Metabolites) (SAM) and Empirical Bayesian Analysis of Microarray (and Metabolites) (EBAM) were used to further identify unique metabolite features between groups.

The peaks-to-pathways function in MetaboAnalyst was used to map metabolite features to relevant pathways. Significant pathways were identified with a false discovery rate (FDR)-adjusted P-value less than 0.05.

Results

Comparison of joint fluid metabolomes between double ACL tear joint fluid and healthy cadaver SF

A total of 1674 metabolite features were detected in injured and healthy joint fluid using LC-MS analysis, with 1241 metabolite features being significantly different between injured and healthy joint fluids (Student's t-test: FDR-adjusted p-value <0.05) (Figure 1A). Similarly, volcano plot analysis showed that 1050 metabolite features were significantly upregulated in joint fluid from an injured individual in comparison to SF from a healthy patient, and 191 were significantly downregulated in injured joint fluid compared to healthy (Figure 1B).

Differences between joint fluids from injured and healthy individuals' joints were further exemplified using unsupervised clustering analyses, including principal component analysis (PCA) and hierarchical cluster analysis (HCA). PCA showed distinct separation between injured and healthy individuals, with PC1 and PC2 accounting for 81.2+5.5% of the variability between data sets (PC1 = 81.2%, PC2 = 5.5%, PC3 = 4.5%) (Figure 2A). Hierarchical cluster analysis further supports differences in joint metabolomes, illustrating clear separation between the metabolomes (Figure 2B). Taken

together, Student's T-test, volcano plot analysis, PCA, and HCA suggest that the metabolome of an injured joint is significantly different from the metabolome of healthy joints.

Metabolite identification and potential discriminatory metabolites

Metabolite features identified as significant or important by these analyses were matched with known metabolite identities. All five statistical analyses identified 616 metabolite features as significant (or important) and matched to 554 metabolite identities. Importantly, all 616 metabolite features were identified as significant by EBAM (Figure 1D). Due to the large number of significant metabolite features, we examined the top 25 m/z values sorted by significance (FDR corrected p-value <0.05). These matched to 11 KEGG compounds: Acetyldihydroliipoamide, Dehydrospermidine, L-Carnitine, Noradrenaline, Dopamine, L-Metanephrine, 3-Hydroxylicocaine, Homovanillin, Homovanillate, 3-Methoxy-4-hydroxyphenylglycolaldehyde, and Vanillylmandelic acid (Figure 1F). Further, these metabolite features matched to 8 KEGG pathways to get an understanding of what metabolic pathways these metabolites played a role in. The affected pathways include: tyrosine metabolism, cAMP signaling pathway, drug metabolism – cytochrome P450, Isoquinoline alkaloid biosynthesis, belatain biosynthesis, thermogenesis, bile secretion, and Dopaminergic synapse.

Pathway identification

The unique metabolite features detected by EBAM resulted in 88 enriched pathways when analyzed by the MS peaks-to-pathways function in MetaboAnalyst. Of these pathways, four were identified as significant (FDR-corrected p-value < 0.05): urea

cycle/amino group metabolism; arginine and proline metabolism; glycerophospholipid metabolism; and lysine metabolism (Figures 3 and 4).

VIP scores from PLS-DA identified the top 25 metabolite features contributing to principle component 1 (Figure 1C). These scores were cross-referenced with the matched compounds from metabolite features that were also recognized as significant by a Student's t-test and identified through the MS peaks-to-pathways function in MetaboAnalyst. The top 25 VIP metabolite features mapped to 31 KEGG compound IDs. Twenty seven of the IDs matched to pathways in KEGG, including amino acid metabolism (phenylalanine, arginine, proline, pyrimidine, alanine, aspartate, and glutamate), Aminoacyl-tRNA biosynthesis, ABC transporters, drug metabolism – cytochrome P450, cyanoamino acid metabolism, arginine biosynthesis, vitamin B6 metabolism, and biosynthesis of alkaloids derived from shikimate pathway (Table 1).

Discussion

This is the first study to use LC-MS-based global metabolomic profiling to understand a patient's joint fluid phenotype subsequent to a second ACL tear. Other studies have used metabolomics to study SF or other tissues following joint injury, but this is the first to assess SF following a second acute joint trauma – a unique sample in which the joint metabolome is very likely to exhibit early signs of PTOA. We identified 11 potential discriminatory metabolites and four enriched pathways which may help to better understand the biochemical response following a second acute joint injury.

Potential discriminatory metabolites

We identified 11 possible discriminatory metabolites of potentially early-PTOA following a second ACL tear. Acetyldihydroliipoamide, Homovanillin, Homovanillate, 3-

Methoxy-4-hydroxyphenylglycolaldehyde, and Vanillylmandelic acid are involved in amino acid metabolism, especially Tyrosine metabolism. Carnitine, norepinephrine, and L-Meteneprine are involved in energy metabolism (37). Dopamine is involved in reward pathways and motor function. Isoquinoline alkaloid biosynthesis and 3-Hydroxyidocaine are both potentially evidence of drug metabolism.

Enriched pathways

Generally, the most common upregulated pathway identified was amino acid metabolism. Similar studies identified that amino acid metabolism is associated with the development of OA (38, 39). More specifically, a study by Chen et al. used targeted metabolomics of serum to investigate amino acid metabolism in OA patients (38). They found that alanine, aspartate, glutamate, arginine, and proline metabolism were the 5 most significant altered amino acids which is consistent with our results. Glutamate and aspartate are excitatory neurotransmitters in the CNS that also play a role in cell signaling, and upregulation of these have been linked to the development of OA (40-43). Further, glutamate and alanine are associated with aggrecan Alanine-Arginine-Glycine-Serine (ARGS) in human SF, which is associated with OA (43, 44). Arginine seems to help bone develop by supporting the deposition of collagen, potentially indicating joint repair following this patient's second ACL tear (45). Tyrosine metabolism could be associated with nitrotyrosine. Nitrotyrosine is a marker of arthritis and joint injury (46) due to its connection to oxidative stress, an important factor in OA (47-49). These results suggest heightened amino acid metabolism in potential early PTOA and following a joint injury.

Further, upregulation of the urea cycle/amino group metabolism pathway could be attributed to oxidative stress, either from the first or second ACL tear. The urea cycle is needed to convert ammonia to urea. Carlson et al. (2018) conducted a similar study using global metabolomics with synovial fluid in OA patients and found upregulation of the urea cycle depending on OA grade (34). Additionally, a systematic review of urea cycle disorders and oxidative stress found that the two are linked (50), possibly showing that this patient has early PTOA.

Malfunction of the urea cycle is, in part, evidenced by excess arginine plasma levels (51, 52), explaining why this pathway was enriched in addition to the urea cycle. However, a review on the enzymes involved in the urea cycle found that arginine deficiency corresponded to excess amino acid catabolism resulting from inflammation or injury (53). Either way, arginine and the urea cycle are tied to oxidative stress which occurs in OA and provides insight to the biochemical response following this double ACL tear.

Lysine metabolism is also potentially indicative of oxidative stress. A study administering excess lysine in mice showed decreased antioxidant activity in the brain (54). Further, a study in humans looking at biomarkers of metabolic syndrome caused by oxidative stress in plasma and urine found that lysine shows promise as a biomarker of oxidative stress (55). Again, oxidative stress could represent early PTOA after a double ACL tear in this patient.

Glycerophospholipids are an important feature of the phospholipid bilayer, so they are present in all cell membranes. Upregulation of glycerophospholipids following a

second ACL tear could be evidence of cellular damage caused by acute joint trauma, although there is no past evidence confirming this connection.

Further, a few of the pathways give insight into possible patient treatment following a second ACL tear. ATP-binding cassette (ABC) transporters help move drug metabolites into cells (56). Likewise, isoquinoline alkaloids are an important component of morphine. They are derived from Tyrosine or DOPA to create dopamine and ultimately isoquinoline alkaloids. Similarly, cytochrome P450 for drug metabolism helps activate many drugs (57). There is also evidence that it is a precursor to L-DOPA, meaning that it could be aiding in isoquinoline alkaloid biosynthesis (58). The pathway for biosynthesis of alkaloids derived from shikimate pathway, which includes isoquinoline, was upregulated in the joint fluid from the injured patient (58). Betalains are also tyrosine-derived plant compounds, although their connection to joint trauma and PTOA is uncertain (58). Together, these upregulated pathways might represent medication prescribed following joint trauma prior to surgery.

Metabolic phenotype of a second ACL tear

In summary, the pathways described in this study give insight into the biochemical response that accompanied a second ACL tear in one patient. Generally, the metabolic phenotype following a second ACL tear included increased evidence of oxidative stress, attempts at rebuilding or restructuring damaged tissue, and amino acid metabolism.

Limitations

There are several limitations in this study that are important to recognize. First, this is a case report of a single unique sample, so the results are a snapshot of one

patient's joint fluid following a second ACL tear and have little generalizability. Future studies should seek to increase the sample size to take into account patient variability and better represent the overall population. Additionally, although the patient has a high probability of getting PTOA due to the nature and repetitiveness of his injuries, it is unknown if he has or will develop it. The altered amino acid metabolism suggests that the patient's joint metabolome is comparable to that of early OA, but this could also represent a normal response to a traumatic joint injury. Further studies are needed to compare the acute response to injury in individuals that will develop PTOA and those that will not.

Moreover, the sample from the injured patient was only compared to healthy postmortem SF, so the injured joint fluid contained, among other components, blood, due to the traumatic injury to the joint. This is not unreasonable, for haemarthrosis is a reported factor in PTOA pathogenesis following ACL tear (8, 59). Likewise, it is unknown if the metabolites and pathways are residue from the first ACL tear or in immediate response to the second ACL tear. Future research should compare other samples like this to joint fluid after one ACL tear, as well as to healthy SF from living patients. Nevertheless, healthy postmortem SF is comparable to living healthy SF if harvested in the appropriate amount of time (60-63), suggesting that this is a reliable comparison. Finally, future studies with untargeted metabolomics need to confirm identities of metabolites with targeted metabolomics to increase the accuracy of our results.

Conclusion

We identified 11 potential discriminatory metabolites of early PTOA and four major enriched pathways. These results potentially provide insight into the biochemical response immediately following a second ACL tear in a single patient.

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Figures

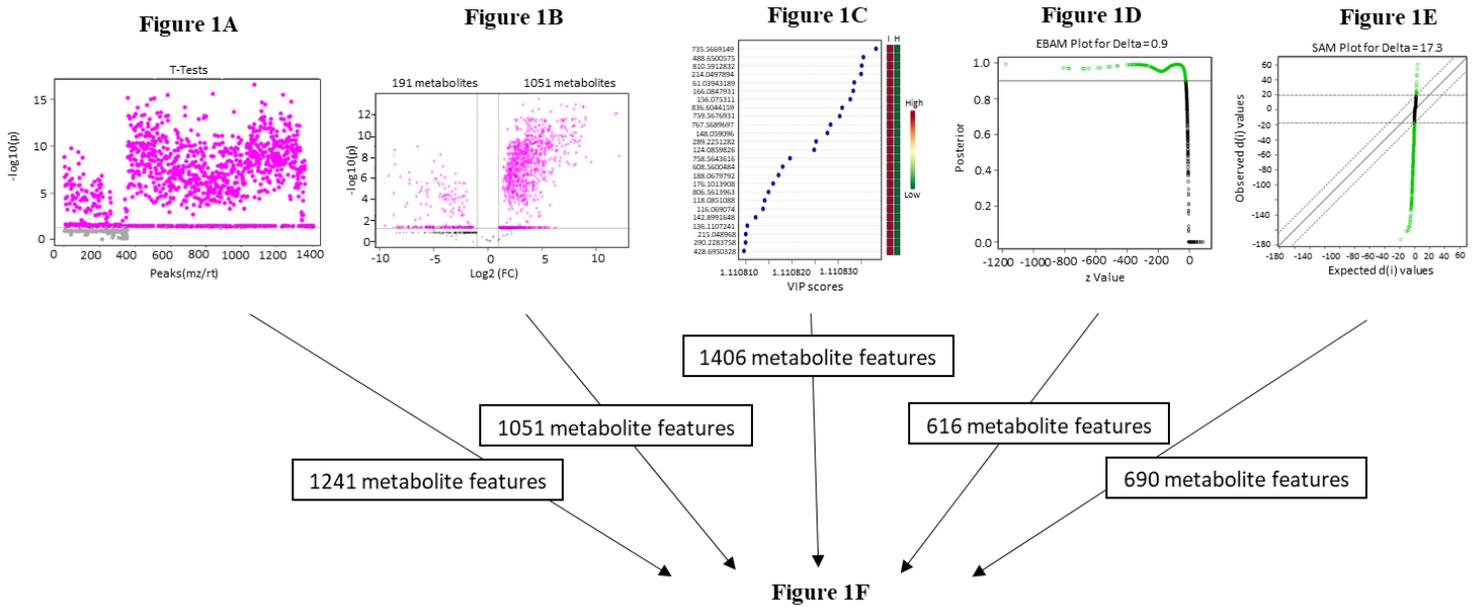


Figure 1. Summary of significant metabolite features in joint fluid following a single patient's second ACL tear compared to healthy postmortem synovial fluid from liquid chromatography-mass spectrometry (LC-MS)-based global metabolic profiling. **1A.** A Student's t-test yielded 1242 significant metabolite features. **1B.** Volcano plot analysis showed that 1051 metabolite features were significantly upregulated and 191 metabolite features were significantly downregulated in joint fluid from an injured patient. **1C.** PLS-DA identified 1406 metabolite features. PLS-DA VIP scores reported the top 25 significantly different m/z values contributing to principle component 1. **1D.** EBAM identified 616 unique metabolite features. **1E.** SAM identified 690 unique metabolite features. **1F.** A table of potential discriminatory metabolites. The unique m/z values were sorted by significance and the top 25 most significant were searched for known KEGG compounds. These matched to 11 KEGG compounds as identified by the table.

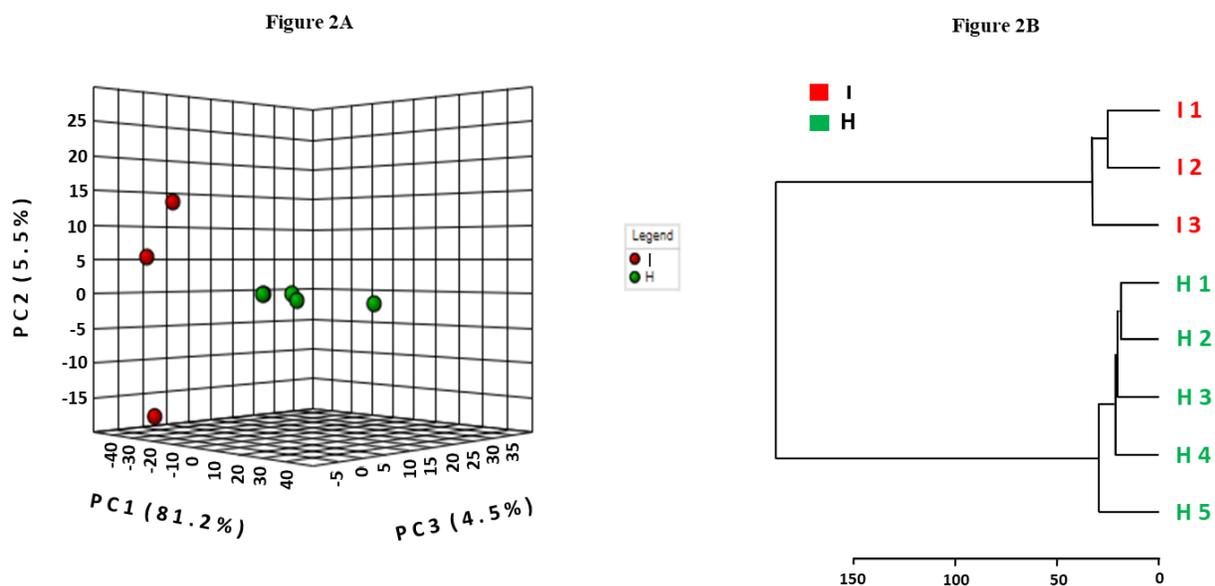


Figure 2. Unsupervised clustering analyses demonstrated differences between injury joint fluid following a single patient’s second ACL tear and healthy postmortem synovial fluid using liquid chromatography-mass spectrometry (LC-MS)-based global metabolic profiling. **2A.** Principle component analysis (PCA) showed distinct separation between injured and healthy, with PC1 and PC2 accounting for 81.2+5.5% of the variability between data sets (PC1 = 81.2%, PC2 = 5.5%, PC3 = 4.5%). **2B.** Hierarchical cluster analysis (HCA) further exemplified differences in joint metabolomes, illustrating clear separation between injured and healthy joint fluid samples.

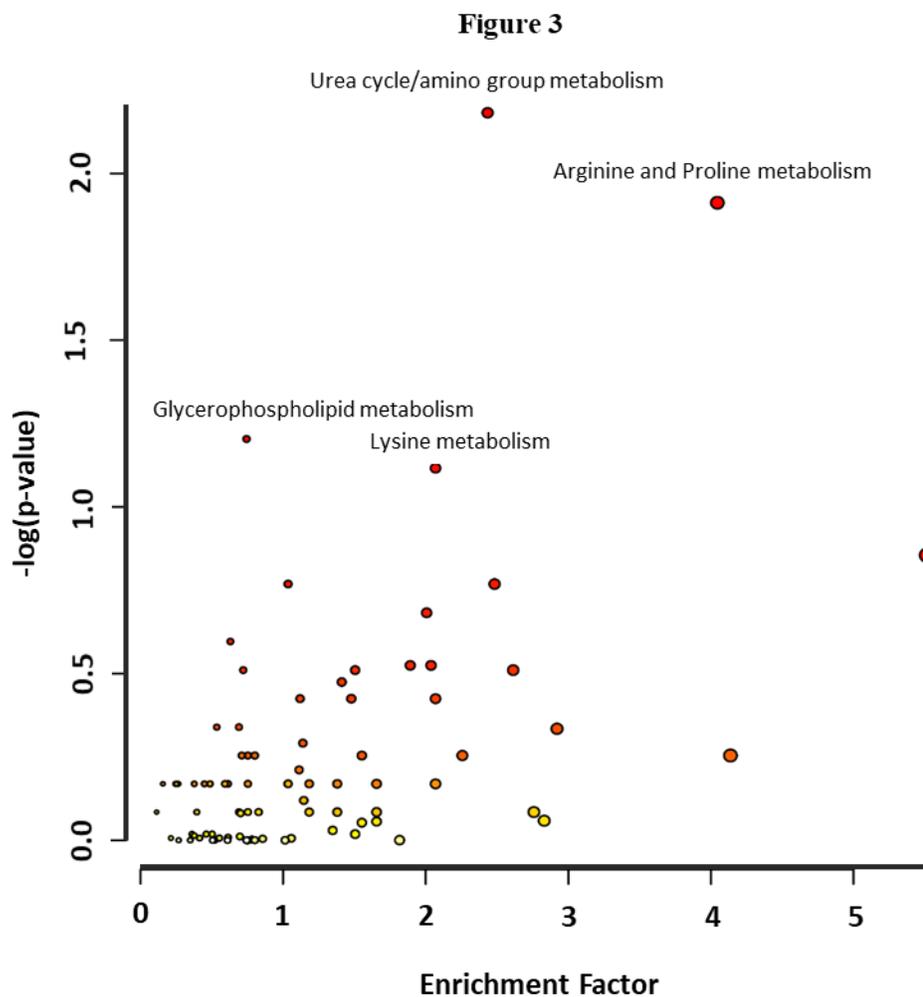


Figure 3. Joint fluid following a single patient’s second ACL tear was compared to healthy postmortem synovial fluid using liquid chromatography-mass spectrometry (LC-MS)-based global metabolic profiling. Four significant enriched pathways (FDR-corrected p-value < 0.05) were identified using Empirical Bayesian Analysis of Microarray (EBAM).

	Identification Source	
	EBAM	PLS-DA VIP Top 25
Pathway	urea cycle/amino group metabolism arginine and proline metabolism lysine metabolism glycerophospholipid metabolism	phenylalanine metabolism arginine metabolism proline metabolism pyrimidine metabolism alanine metabolism aspartate metabolism glutamate metabolism aminoacyl-tRNA biosynthesis ABC transporters drug metabolism – cytochrome P450 cyanoamino acid metabolism arginine biosynthesis vitamin B6 metabolism biosynthesis of alkaloids derived from shikimate pathway

Table 1. Synthesis of significantly upregulated pathways as identified by EBAM and PLS-DA VIP scores Top 25.