Metabolomic profiling of a novel in vitro *model of meniscal tears*

By

TYSEN KIRK TIMMER

The University of Washington School of Medicine

In Conjunction with Montana State University

Seattle, WA
Bozeman, MT

January 2016

Dr. Ron June, Faculty Mentor

Department of Mechanical & Industrial Engineering

Abstract:

Background- The menisci act as one of the main stabilizing and load-distributing structures in the knee joint. They are composed mostly of structured type 1 collagen, proteoglycans, and meniscal fibrochondrocytes and are the most commonly injured structure of the knee. The tissue is only capable of limited self-repair in the case of injury. One exciting new area of research is using metabolomics to study mechanotransduction. This technique quantitatively profiles the processes by which cells respond to mechanical stimuli by biochemical signals that characterize downstream cellular responses.

Objective- The objective was to identify candidate mediators of meniscal fibrochondrocyte mechanotransduction in mouse tissues using *in vitro* organ culture. We aim to uncover metabolomic differences between injured and uninjured tissues and also differences between the tissues in isolation and in combination.

Methods- Mouse knee tissue samples were randomly assigned to isolated or combination experimental groups. The structures were cultured for 12 days with half of the menisci being injured. Metabolites were characterized by HPLC-MS and untargeted metabolomic analysis to examine changes in global metabolomic profiles.

Results- Untargeted metabolomics and cluster analysis revealed changes in 1440 total metabolites, as well as changes in regulation of metabolomic pathways in the response of isolated menisci to injury compared to uninjured isolated menisci. For tissues cultured in combination, similar results were obtained with activation of a larger number of pathways. We discovered a total of 111 metabolic pathways altered by injury. Frequency analysis of individual metabolites in one of the combination samples revealed that the top ten individual metabolites are present in a majority of the upregulated pathways.

Conclusions- These data show that metabolomic differences exist between injured and uninjured meniscal fibrochondrocytes, and also metabolomic differences exist when comparing the response of these injured isolated samples to that of injured samples in combination with other knee tissues.

1. Introduction-

The knee is the largest joint articulation in the body. It has an extensive range of motion due to its modified hinge design. The stability of the joint is provided by the soft tissue structures including the anterior and posterior cruciate ligaments, medial and lateral collateral ligaments, menisci, synovium, and others. 10-15% of adults in the United States report knee symptoms with over 3.3 million new visits each year (Praemer et al.,1999). Overall, knee pain accounts for 3% to 5% of all visits to physicians, and a large proportion result in diagnostic imaging and referral to specialty care (Katz et al., 2000).

Meniscal injuries are the most common soft tissue injury of the knee. The two menisci contained within the joint are crescent pads of fibrocartilage which are located between the femoral condyles and the tibial plateaus (McNulty et al., 2015). As shown in Figure 1, the menisci are wedge-shaped from a cross sectional view. Their thicker portions are along the joint periphery, where they attach to the synovial joint capsule of the knee. The medial meniscus is attached to the medial collateral ligament, whereas the lateral meniscus isn't attached to the lateral collateral ligament. The lateral meniscus is therefore more mobile, which may contribute to its lower injury rate compared to the medial meniscus. The function of the menisci is to dissipate loading forces placed on the knee, stabilize rotation, and lubricate the joint. Acute meniscal tears often occur from twisting injuries. They can also be chronic degenerative tears that occur with minimal stress in older patients (Binfield et al., 1993). Meniscal injuries can occur in isolation or in association with other soft tissue injuries of the knee. If left untreated, large complex tears impair smooth motion of the knee joint, lead to osteoarthritis, and can cause joint effusions. In addition, the menisci demonstrate little capacity for self-repair, except for a small percentage of injuries occurring in the peripheral vascularized region (Scott et al., 1986). Partial meniscectomy is often utilized to treat these injuries. This can result in improvements in pain and function but is not protective against the development of osteoarthritis (Andersson-Molina et al., 2002, Hall et al., 2014).

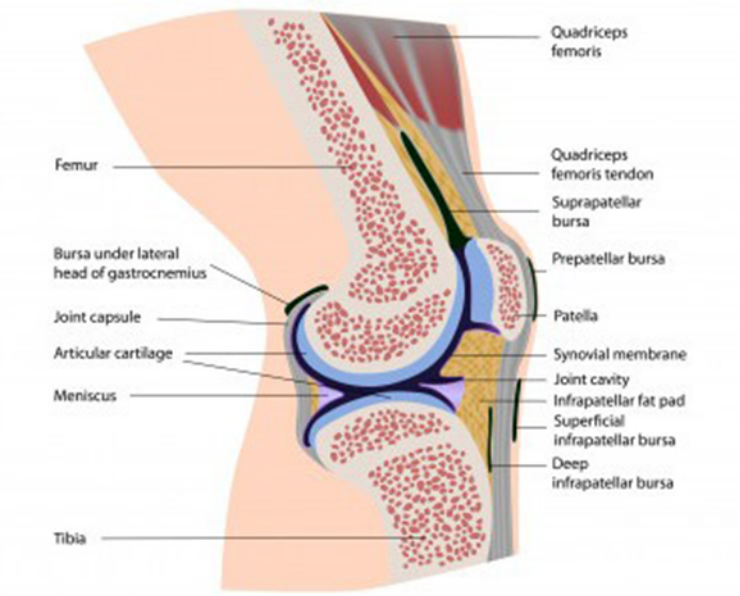


Figure. 1. A diagram showing the knee in cross sectional view. Illustrating how the structures of the knee joint interact to provide a stable yet mobile joint. Structures to note include the joint capsule, synovium, meniscus, articular cartilage, femur, tibia, as well as other ligaments and soft tissue structures. Image from http://www.kneepaindiagnosis.info/.

One promising new area of research regarding knee injuries and disease is mechanotransduction. Mechanotransduction refers to the processes by which cells sense and respond to mechanical stimuli by converting them to biochemical signals that elicit specific cellular responses. These events are vitally important in response to an injury, and knowing more about them provides important insights into the treatment and even prevention of knee injuries and degeneration. Prior studies have elucidated initial mechanotransduction pathways of chondrocytes within the knee (Jutila et al., 2014). The feasibility of utilizing mass spectrometry analyzation of metabolomics as a tool for understanding chondrocyte mechanotransduction has also been demonstrated. This research has revealed correlations of increased mechanical loading and central energy reorganization in osteoarthritis and identified several metabolites that could potentially be mediators for chondrocyte mechanotransduction via energy, lipid, and amino acid metabolism (Jutila et al., 2014 and Zignego et al 2015).

While the metabolomic response of chondrocytes has been described, meniscal fibrochondrocyte mechanobiology has received comparatively little attention, and the

mechanisms involved are still not well elucidated. Many of the mechanotransduction pathways examined in meniscal cells are based upon those found in articular chondrocytes. The menisci are maintained via a balance of the anabolic and catabolic activities of the meniscal fibrochondrocytes. The biological activities of meniscal fibrochondrocytes has been shown to be influenced by biochemical factors, such as cytokines or growth factors (McNulty et al., 2013 and Riera et al., 2011). However, research in this field is still in its infancy. Studies have focused mainly on *in vivo* animal studies which are limited by the complexities of defining the specific mechanical environment of the menisci (Verdonk et al., 2005). On the tissue level, *in vitro* studies have discovered important information on the metabolomic regulation of meniscal cells. However, these studies have focused on isolated cell cultures grown in a three dimensional matrix (Gupta et al., 2008 and Zielinska et al., 2009). This focus on a single cell type cannot account for interactions between tissues of different type as would be the case in a true biological model. Using untargeted metabolomics, we aim to use partial and complete soft tissue samples both in isolation and in combination to identify candidate mediators of meniscal cell mechanotransduction in mouse knees. We hypothesize that there will be metabolomic differences between injured and uninjured meniscal fibrochondrocytes in isolation, and also differences between the samples in isolation and in combination with other tissues. These studies add to the understanding of the mechanobiologic responses of the meniscus and other knee tissues. Extension of this work may lead to novel therapeutic approaches to enhance repair and regeneration of the meniscus.

2. Materials and Methods-

2.1 Mouse selection-

For this study, healthy male C57 mice at 8 weeks age were selected for tissue harvest and culture. The mice were randomly assigned to one of seven groups (n=3). The study was designed to answer two main questions: does a meniscal injury alter the metabolomic profiles of meniscal fibrochondrocytes in isolation? And how does this response compare to the injury response while in culture in combination with other knee

tissues? Table 1 lists specific groups that were cultured for this study. To assess potential background metabolites, a negative control of fresh media was also examined.

Table 1
Mouse tissue harvest samples. Samples were selected to compare both injured vs uninjured as well as isolated vs in combination. Each sample resided independently in a single well of a 24 well plate for tissue culture, and metabolites were extracted at the termination of the study. The purpose of this study was to learn more about meniscal injuries. Therefore, uninjured meniscus, injured meniscus, uninjured combination, and injured combination samples were then analyzed via LC-MS. Synovium, tibial plateau, and femoral condyle isolated samples were cultured and saved for future experimentation regarding their injury characteristics in isolation.

Sample Type	Sample ID
Femoral Condyles	723F1, 723F2, 723F3
Tibial Plateau	723T1, 723T2, 723T3
Uninjured Meniscus	723MU1, 723MU2, 723MU3
Injured Meniscus	723MI1, 723MI2, 723MI3
Synovium	723S1, 723S2, 723S3
Uninjured combination	724CU4, 724CU5, 724CU6*
Injured Combination	723CI1,723CI2, 723CI3
Negative Control	NC

*a single combined tissue sample was withheld and preserved for future histological analysis

2.2 Tissue harvest-

Prior to the study, we developed a novel technique for harvesting undamaged and sterile menisci, synovium samples, tibial plateaus, and femoral condyles from the mice. In our laboratory, this can be performed without contamination of the individual tissues. The advantage of this technique is that it allows experimental tissue culture of isolated and/or combinations of joint tissues during experimental manipulations. For an in depth pictured procedural tutorial of this process please see supplemental Figure 1. First, mice were humanely euthanized using carbon dioxide. To prevent bacterial

contamination, all tools (forceps, scalpel, and small scissors) had been previously sanitized using 70% ethanol. To harvest the joint structures, the euthanized mice were first liberally sprayed with 70% ethanol and placed onto a sterile surface for harvest. An incision is made through the skin on the posterior side of the mouse at about the mid lumbar region using small sterile scissors. The incision is then extended to completely circumflex the mouse at this level. Using a sterile gloved hand, the skin of the mouse is then pulled inferiorly separating it completely from the body of the mouse and fully exposing the knee joint for harvest. Using small scissors the upper leg muscles are cut distally to proximally staying parallel and as close as possible to the femur. Several cuts may be needed. Then the muscles can be completely freed from the joint. Any small bits of muscle left over can be removed by scraping with a scalpel and/or tweezers. A similar process is performed on the inferior side of the joint. The incision is made through the gastrocnemius, and the lower leg muscles are removed resulting in a joint free from all musculature. The next step is to harvest a 2mm X 4mm synovium sample from lateral side of the joint using a scalpel and forceps. The sample is immediately rinsed in sterile phosphate buffered saline (PBS) and then placed in a 24 well plate in 400um culture media. Next, all external connective tissue (LCL, MCL, Patellar tendon, Hamstring tendons) are severed using scissors. Using traction inferior to the joint to open up the joint space, the joint is separated using a sharp scalpel through the ACL and PCL. It is important at this step to provide proper joint space so as not to damage the menisci and/or articular cartilage on either the tibia or humerus. Once the joint is separated the menisci are carefully removed from the tibial plateau using forceps and small scissors. They are then rinsed in sterile PBS and placed in culture media in the 24 well plate. The final steps are to remove both the tibial plateau and the femoral condyles from the long bones. This is facilitated by using sharp small scissors and ensuring to cut on the joint side of the epiphyseal plates. These samples are washed in PBS and placed into media in the 24 well plate for culture. As noted in Table 1, extra isolated tissue samples of other knee tissues were obtained for future isolated experimentation. The current study aims to analyze the characteristics of meniscal injuries in isolation and in combination with other tissues, but not yet the other tissues alone. To denote groups, we use “isolated” to denote samples cultured individually (*e.g.* a single

meniscus) and “combination” to denote samples cultured together (*e.g.* meniscus, synovium, articular cartilage, and subchondral bone.)

2.3 Media preparation and organ culture-

In order to optimize metabolite analysis, three separate types of culture media are used in this experiment. Samples were originally cultured in Dulbecco’s modified eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics (10,000 I.U./mL penicillin and 10000 µg/mL streptomycin) at 37° C and 4% atmospheric carbon dioxide. As illustrated by Supplemental Figure 2, the experimental timeline involves multiple media exchanges. Fetal bovine serum is an essential ingredient for long term tissue culture. However, being an animal product, it contains several metabolites, which complicate the metabolomic profiling experiments. To avoid this problem, after a 7 day equilibration period in the FBS media, the culture media was switched to media containing DMEM with 10% insulin-transferrin-sodium selenite media supplement (ITS) and antibiotics (10,000 I.U./mL penicillin and 10000 µg/mL streptomycin). To further rule out the antibiotics effect on the metabolomics data, the media was switched again at day 11 to media containing only DMEM with 10% ITS supplement. At this time, half of the isolated meniscus samples and a single meniscus in three of the combination samples were also injured. This was done using small scissors to inflict a generalized partial meniscal tear. Metabolites were extracted as described below on day 12. All media changes were done in sterile conditions under the hood using a 1000um pipet and sterile pipet tips. Existing media was aspirated off and replaced with 500um PBS to wash the sample. This was repeated twice before adding in the new FBS free media.

2.4 Metabolite extraction-

Metabolite extraction was performed using a modified version of methods perfected by Zignego *et al*. First, each sample was transferred into a sterile 1.5ml microfuge tube (RNA quality). 1ml buffer was then added (70:30 (v/v) Methanol:Acetone, HPLC grade) and the tubes were placed in a -20° C freezer for 5 minutes. The tubes were taken from the freezer and vortexed and then placed back into the freezer for four total cycles. After keeping in the freezer overnight, a final vortex was performed before removing solids and macromolecules by centrifugation at max rpm at 4°C for 10 minutes. Supernatant

was transferred to a fresh tube, and solvent was evaporated using a speed vacuum centrifuge for 8 hours. Finally, samples were stored at -20 overnight, and the following day dried samples were then resuspended in 100 µL of mass spectrometry grade water and acetonitrile (50:50 v/v) for LC-MS analysis.

2.5 Untargeted metabolomic profiling-

Metabolomics refers to an experimental technique used to characterizing a large number of small molecules in biological samples (Patti et al., 2012). Few studies have employed this technique to examine meniscal fibrochondrocytes or identify candidate biomarkers in their cellular responses. In this study, metabolites were extracted following culture of knee tissues and analyzed via nano-liquid chromatography and mass spectrometry. To detect untargeted metabolites we used positive mode on an Agilent 6538 Q-TOF spectrometer with an accuracy of ~5 ppm and a resolution of ~20,000.

2.6 Compound identification and enrichment analysis-

In order to identify compounds, a batch search of all of the untargeted metabolite mass to charge (m/z) values was done using METLIN which is a database that contains over 14,000 identifiable metabolites (Wishart et al., 2013 and Zhu et al., 2013). Our search parameters included a mass tolerance of 15 ppm, and positively charged molecules with potential +1H+ or +1Na+ adducts. MZMine2.14 was used to set a minimum threshold of 1000 for m/z value intensity. After compound identification, untargeted metabolites were examined using medians of each group (n=3) and performing unsupervised clustering. This allows identification of groups of co-regulated metabolites. These clusters containing co-regulated metabolites were analyzed using IMPaLA for pathway enrichment. IMPaLA performs enrichment analysis using specified lists of metabolites and over 3000 pathways from 11 databases (Kamburov et al., 2011). Due to the inherently large data sets obtained with mass spectrometry, false positives are a real concern. P-values even of 0.05 often fall short of excluding these false positives. Instead, a Q-value cutoff of 0.25 was used. A Q-value is an adjusted P-value ideal for large data sets that is calculated using an optimized FDR approach.

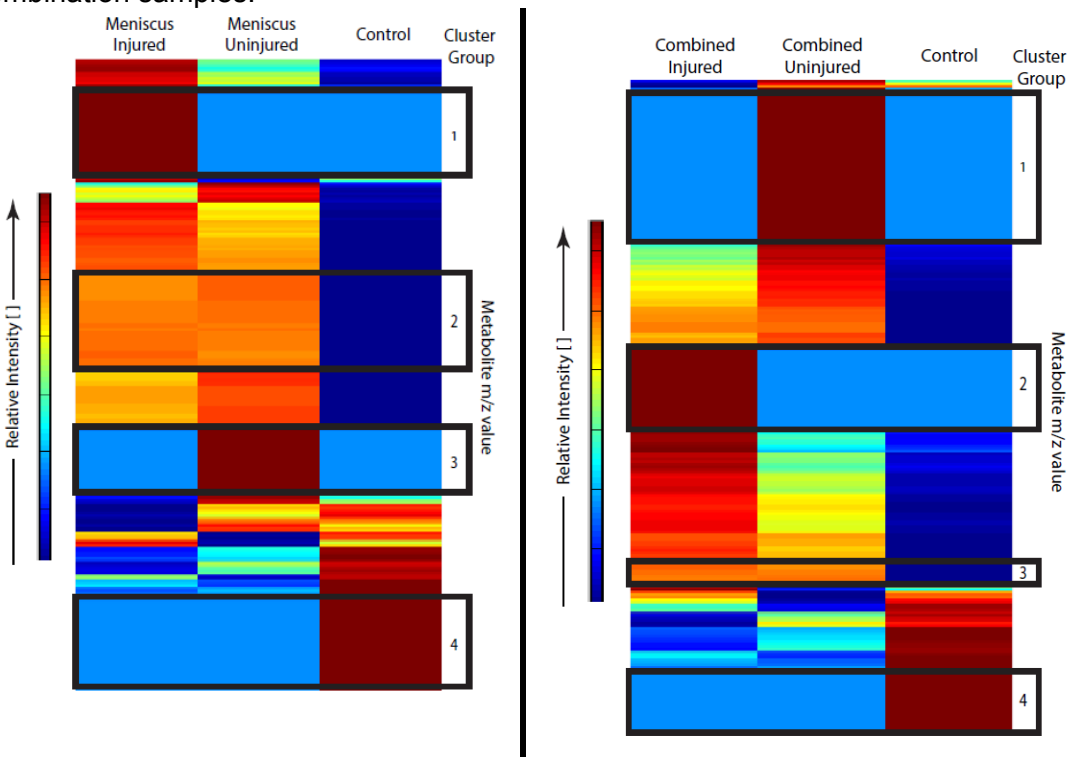
3. Results-

The objective of this study was to characterize the cellular response to injury of meniscal fibrochondrocytes both in isolation and in combination with other knee tissues. In order to minimize bias from selecting only certain pathways, we analyzed untargeted metabolomics profiles which prevents the *a priori* exclusion of potentially important data. Our data demonstrate that injury alters the untargeted metabolomic responses of isolated menisci when compared to uninjured isolated menisci. Also, there is limited crossover and a much more simplistic response when comparing the untargeted mechanobiology of an isolated injured meniscus to that of an injured meniscus cultured in combination with other knee tissues.

3.1 Untargeted Analysis-

We detected several thousand metabolites in each of the data sets. These untargeted metabolites were refined using unsupervised clustering which identified eight total clusters, of which four were of interest for comparison for metabolomic differences (Figure 2). To determine the effects of injury on meniscal fibrochondrocyte metabolism, injured isolated menisci were first compared to isolated uninjured and negative controls. This comparison revealed changes in 1440 metabolites (760 independent to MI, 680 MU). There were 3060 different metabolites between the injured and uninjured combination samples (2013 CI, 1047 CU). When comparing the isolated injured meniscus to the injured combination sample we discovered 3206 different metabolites (1543 CI, 1663 MI).

Figure 2.
Heat maps of unsupervised clustering analysis showing upregulated (dark red) samples and demonstrating similarities and differences both between samples and separate clusters. Isolated meniscus samples on the left and combination samples on the right. For this study we focused on cluster 1 and 3 in isolated meniscus samples and clusters 1 and 2 from the combination samples.



3.2 Enrichment Analysis-

Using strict Q-value cutoffs to minimize false positives, enrichment analysis of the four selected clusters identified a total of 111 pathways that were either up or downregulated in the samples. These pathways reveal differences in the tissue phenotypes due to injury and combination culture. Specifically, meniscus cluster 1 revealed nine total pathways that were upregulated in the injured sample compared to the uninjured sample and negative control (Table 2). Whereas meniscus cluster 3 showed nine total pathways that were downregulated in the isolated injured meniscus sample as compared to the uninjured and negative control (Table 2). The data for combined cluster 1 reveal only a single metabolic pathway was downregulated in an uninjured combination sample when compared to combination injured and negative control samples (Table 2). In contrast to this, the combined cluster 2 revealed a total of 92

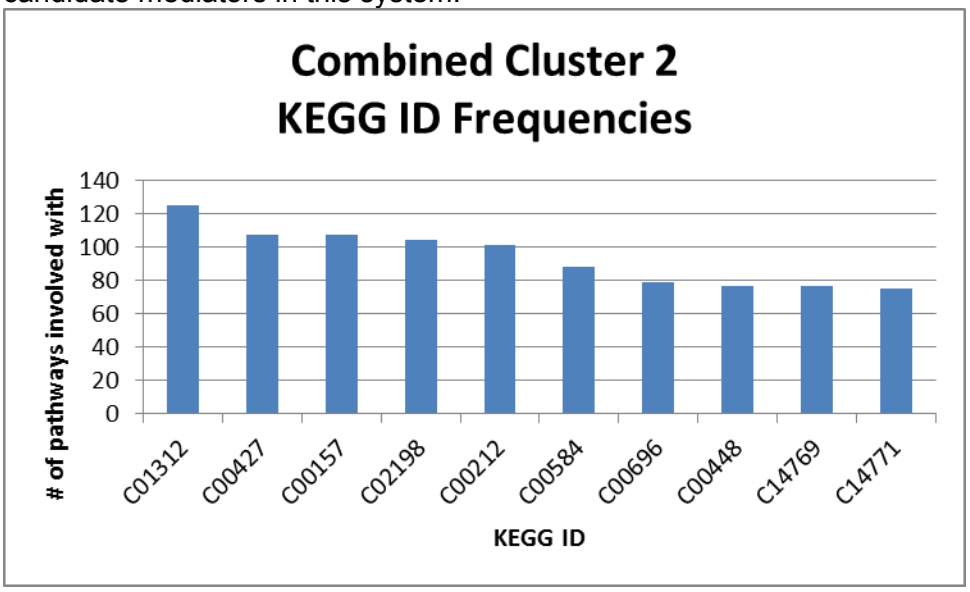
metabolic pathways were upregulated in the injured combination sample when compared to negative control and uninjured combination samples. As shown in table 2, when comparing common pathways between clusters, the only overlap occurs between Meniscus Cluster 1 and Combined Cluster 2. There were no common pathways between the remaining clusters.

Table 2.
This table displays the up and downregulated pathways present in the four examined clusters when examined by enrichment analysis. Combined cluster 2 was further restricted to the top 10 metabolites for the purposes of this study.

Cluster	Pathway	Mechanosensitive metabolites [KEGG ID]
Meniscus Cluster 1	C20 prostanoid biosynthesis	C00427;C00584;C00696;C02198;C01312
	Quercetin and Nf-kB- AP-1 induced cell apoptosis	C00427;C00584;C00696;C02198;C01312
	Prostanoid ligand receptors	C00584;C00696;C02198;C01312
	Drug metabolism - Cytochrome P450	C16571;C07492;C07501;C16654;C07496;C11785;C16648;C16548;C16653;C16608;C16606;C16602;C16601
	Synthesis of Prostaglandins and Thromboxanes	C00696;C01312;C04671;C02198;C00427;C05960;C07589;C00584
	Eicosanoid metabolism	C00427;C00584;C00696;C01312
	Eicosanoid Synthesis	C00427;C00584;C00696;C02198;C01312
	Arachidonic acid metabolism	C00696;C01312;C04671;C05960;C00427;C02198;C07589;C06314;C06315;C04853;C00584
	Eicosanoid ligand-binding receptors	C00584;C00696;C02198;C01312
Meniscus Cluster 3	Facilitative Na+ independent glucose transporters	C10906;C00124;C00095;C00267
	Galactose metabolism	C00159;C00116;C00795;C05404;C00267;C00095;C00492;C00124;C00137
	Digestion of dietary carbohydrate	C10906;C00267;C00095;C01835;C00124
	Na+ dependent glucose transporters	C00159;C00124;C00267
	Class II GLUTs	C10906;C00267;C00095
	ABC transporters	C00503;C00159;C00116;C00095;C00079;C06687;C04137;C04114;C16692;C00492;C00137;C01835;C00135
	Transport of glucose	C00267;C00159;C10906;C00095;C00079;C00082;C07151;C00124;C00137;C00135
	Starch and sucrose metabolism	C02336;C00267;C03033;C00095;C06219;C00221;C00721;C06215
	Estrogen metabolism	C00468;C05298;C03033;C05300
Combined Cluster 1	Linoleic acid metabolism	C07338;C00157;C19937;C07354;C14836;C14837;C14835;C14833;C14831
Combined Cluster 2	Arachidonic acid metabolism	C04230;C00696;C01312;C04671;C05952;C05963;C05960;C14769;C06314;C00427;C02198;C05961;C04805;C14771;C06315;C04853;C00157;C00584
	Eicosanoid Synthesis	C00696;C01312;C05952;C05963;C02198;C00427;C04805;C00584
	Ketoprofen Action Pathway	C04230;C00696;C01312;C05961;C01716;C05963;C02198;C00157;C00427;C04805;C14771;C04853;C14769;C00584
	Antipyrine Action Pathway	C13244;C04230;C00696;C01312;C05963;C02198;C00157;C00427;C04805;C14771;C14769;C04853;C05961;C00584
	Phenylbutazone Action Pathway	C14769;C04230;C00696;C01312;C04805;C05963;C02198;C00157;C00427;C07440;C14771;C04853;C05961;C00584
	Rofecoxib Action Pathway	C14769;C04230;C00696;C01312;C04805;C05963;C02198;C00157;C00427;C07440;C14771;C04853;C05961;C00584
	Leukotriene C4 Synthesis Deficiency	C04230;C00696;C01312;C05952;C05963;C02198;C05961;C00427;C04805;C06314;C04853;C00157;C00584
	Antrafenine Action Pathway	C14769;C04230;C00696;C01312;C05963;C02198;C00157;C00427;C04805;C14771;C04853;C05961;C00584
	Lumiracoxib Action Pathway	C14769;C04230;C00696;C01312;C05963;C02198;C00157;C00427;C04805;C14771;C04853;C05961;C00584
	Trisalicylate-choline Action Pathway	C14769;C04230;C00696;C01312;C05963;C02198;C00157;C00427;C04805;C14771;C04853;C05961;C00584

To focus on the most significant data, the top 10 upregulated pathways from combined cluster 2 were further analyzed to compare to the other clusters. However, to avoid missing potentially important data and to provide a basis for future studies, the individual metabolites within these 92 pathways were analyzed by their frequency of appearance in multiple pathways. This revealed that the ten most frequent of the 166 unique individual metabolites (identified by KEGG ID) were common to a majority of the 92 total pathways (Figure 3).

Figure 3.
An analysis of the frequency of individual metabolites (KEGG ID) that appeared in combined cluster 2 pathways. Overall, there were a total of 166 unique metabolites present with the top 10 appearing in a vast majority of all pathways. This offers insight to the importance of the particular metabolites on a system wide basis and offers potential for further study of them as candidate mediators in this system.



4. Discussion-

The questions our study asked were: does a meniscal injury alter the metabolomic profiles of meniscal fibrochondrocytes in isolation? And how does this response compare to the injury response while in culture or in combination with other knee tissues? Our *in vitro* results support our hypothesis and show *in vitro* injured and uninjured meniscal fibrochondrocytes exhibit differences in metabolomic profiles. Furthermore, metabolomic profiles are different when comparing the response of injured isolated samples to injured samples cultured in combination with other knee tissues. One limitation of the study is that it cannot be assumed to be as physiologically relevant as an *in vivo* study. Also, our experiment only captures these events on a single timescale, whereas the metabolic response could be different at earlier points in time. Future studies could look at the events on a shorter (minutes to hours) timescale. However, the timescale used in these experiments is a reasonable approximation of the

time it would take for a traumatic meniscal tear patient to reach a physician's office for potential diagnostic analysis of synovial fluid.

Specifically, we cultured menisci in isolation and in combination with other knee tissues and injured half of the menisci. In doing so, we observed changes in thousands of untargeted metabolites. Unsupervised clustering analysis showed upregulated samples and demonstrated similarities and differences both between samples and separate clusters (Figure 2). The metabolomic profile can be interpreted as a description of the cellular phenotype (Patti et al., 2012). In the case of this study, these metabolomic changes signify an altered cellular phenotype as a result of exogenous injury. The ability of metabolomics to portray meniscal fibrochondrocyte responses to injury is highlighted by our observation that, when comparing isolated injured menisci to isolated uninjured menisci there was a difference in 1440 total metabolites. The importance of the presence of other knee tissues in regulating meniscal fibrochondrocyte behavior is further emphasized by the vast increase in total number of different metabolites when comparing isolated injured menisci samples to injured menisci samples in combination. These large-scale differences demonstrate the potential importance of the interaction with other knee tissues in the process of meniscal fibrochondrocyte response to injury, and provides a multitude of candidate mediators in meniscal fibrochondrocyte mechanotransduction. Future studies may build on this untargeted dataset by using targeted models using these candidate mediators and looking at specific pathways of interest.

In addition, we identified mechanosensitive cellular pathways by using enrichment analysis of clusters of co-regulated metabolites (Figure 2, Table 2). Injury notably increased activation of several pathways in isolated menisci samples when compared to uninjured isolated and negative controls (Figure 2, Table 2). These pathways included eicosanoid synthesis and metabolism, arachidonic acid metabolism, synthesis of prostaglandins and thromboxanes, apoptosis pathways, and others (Table 2). The most striking finding is that seven of the nine upregulated pathways are related to eicosanoid hormone production, regulation, or action. The eicosanoids consist of prostaglandins, thromboxanes, leukotrienes, and lipoxins. Arachidonic acid is the immediate precursor

of the eicosanoid hormones. Additionally, prostaglandins and thromboxanes together are termed prostanoids, and it is widely accepted that they are the main mediators of the inflammatory response (Ricciotti et al., 2011). Although very little data has been published investigating the metabolomic response of menisci to injury, it had been demonstrated that menisci upregulate production of prostaglandins when exposed to compression forces (Hennerbichler et al., 2011). It has also been firmly established that these hormones play a major role in the injury and regeneration of articular fibrochondrocytes (Aoyama et al., 2005 and Otsuka et al., 2009). To our knowledge, this is the first study to demonstrate a metabolic upregulation of eicosanoid related pathways as a response to meniscus injury. Future investigation of these pathways in this system could provide valuable input to treatment and prevention of meniscal injuries by pharmacological therapy. One class of drugs particularly interesting in this case would be the NSAIDs since they interact and inhibit these pathways directly.

Several other metabolomic pathways were downregulated in response to isolated meniscus injury when comparing to isolated uninjured and negative controls (Figure 2, Table 2). Of note in this cluster was that seven of the nine downregulated pathways are related to glucose or sugar metabolism and transport (Table 2). This is a very interesting finding, especially when taken in the context of prior studies. Although specific meniscal fibrochondrocyte data is lacking, multiple studies have demonstrated catabolic responses to mechanical injury of articular chondrocytes (Anderson et al., 2011 and Petursson et al., 2013). It has also been shown that physiological loading forces on chondrocytes promote anabolic pathways and upregulation of glucose flux in articular chondrocytes (Zignego et al., 2015). Further investigation into our findings could reveal that the same pathways have implications in not only articular chondrocyte reaction to injury, but also in that of the response of meniscal fibrochondrocytes.

When considering both the upregulated and downregulated pathways in the injured combination clusters, it is interesting to note that only one pathway is downregulated in injured meniscal fibrochondrocytes when compared to uninjured combination and negative controls. On the contrary, a total of 92 pathways are upregulated in these samples. For interpreting this combination data, the absence of a large number of

activated pathways may indicate a homeostatic response in which there are few pathways dominating the normal (e.g. uninjured) meniscal fibrochondrocyte. In contrast, with meniscal injury there is an enormous response in the upregulation of pathways in the presence of other knee tissues. It does seem counterintuitive that the single downregulated pathway happens to be linoleic acid metabolism, since linoleic acid it the precursor for arachidonic acid which is among the dominating upregulated pathways in both isolated and combined injured meniscal fibrochondrocytes (Table 2). This contrast in isolated vs. combination metabolomic responses offers intriguing possibilities for future studies to further elucidate the etiologies of these differences.

Also, of note when comparing the isolated and combination samples is the vast increase in the complexity of the metabolomic response when menisci are cultured in combination with other knee tissues as opposed to isolation. We found an additional 81 significantly upregulated pathways in the combination injured sample as opposed to the isolated injured sample. When comparing the top ten of the upregulated pathways in the injured combined sample, only two overlap with the injured isolation sample (Table 2). Arachidonic acid metabolism and eicosanoid synthesis are upregulated in both samples which points to their significance. However, perhaps the more important point of this observation is that more upregulated pathways are not shared by the two groups. The data support our hypothesis that metabolic differences are present in meniscal fibrochondrocyte response to injury when cultured alone versus in combination with other knee tissues. This insight could help direct future *in vitro* studies to look at meniscal injuries on a system wide scale and not only in isolation.

Lastly, in order to avoid arbitrarily overlooking important data by restricting combined cluster 2 to the top ten pathways, we performed frequency analysis of the individual metabolites common to multiple pathways. This revealed 166 unique metabolites of which the ten most frequent were common to a majority of the 92 total pathways (Figure 3). These data indicate that certain individual metabolites which are represented in a higher percentage of pathways play a more vital role in the system overall. A lack of resources to further investigate each of the 92 pathways is one limitation of this study. Further investigation would be warranted in the future. Alternatively, future projects

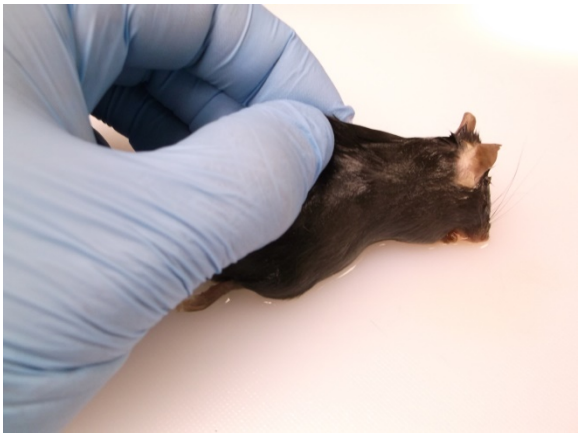
could increase the minimum intensity threshold from 1000 to 2000 during mass spectrometry data analyzation. This would result in fewer positive metabolic results and ensure that the focus remains on those metabolites that are of greatest impact on the system as a whole.

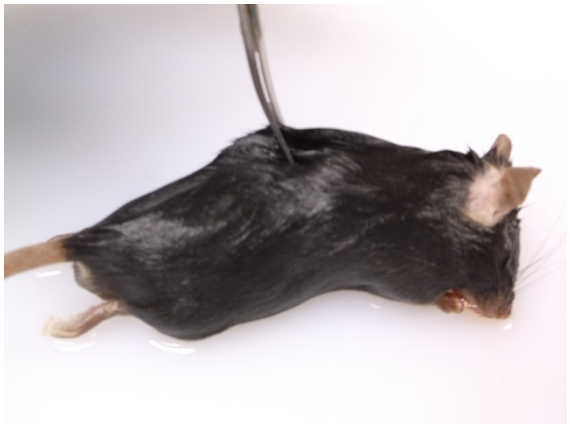
Overall, this study provides new insight into a relatively understudied field. The majority of metabolic inquiry into knee tissues has occurred in the articular chondrocyte. What little research has been done into the metabolomics of meniscal fibrochondrocytes has neglected to look at the cells as part of a system including other knee tissues. Our data provide numerous candidate mediators important to meniscal fibrochondrocyte mechanotrasduction and offers great potential for expansion using targeted metabolomics. Although this is the first report of this *in vitro* model, our data provide interesting possibilities for future research and demonstrates differences in mechanotransduction between injured and uninjured isolated menisci, as well as a difference in this metabolomic response when viewed in isolation or in combination with other important knee tissues. Extension of this work may lead to novel therapeutic approaches to enhance diagnosis, repair, and regeneration of the meniscus.

Supplemental Figure 1.
We created a novel technique to efficiently harvest mouse knee tissue samples. The details of which are laid out below in a stepwise fashion with photos.

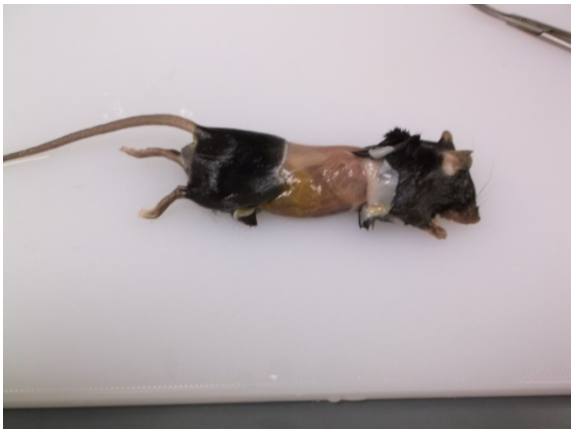
1) Liberally spray mouse with 70% ethanol and place onto a sterile surface

2) Make an incision through the skin on the posterior side of the mouse at about the mid thoracic region using small sterile scissors.

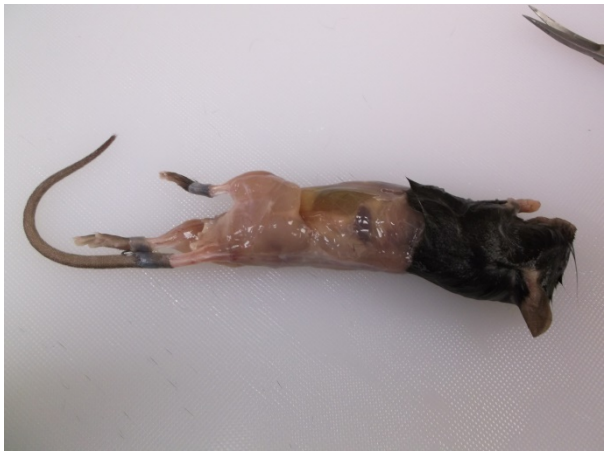




3) Extend the incision to circumflex the mouse at this level.

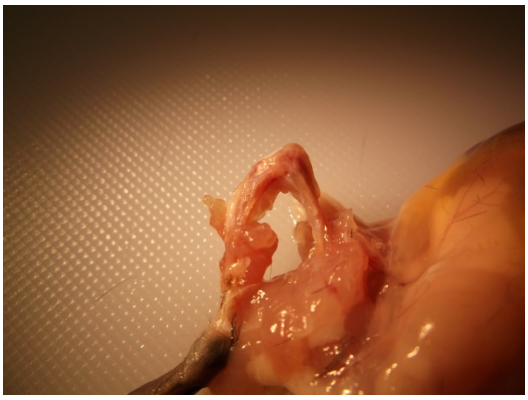


4) Using a sterile gloved hand, pull the skin of the mouse inferiorly separating it completely from the body of the mouse and fully exposing the knee joint for tissue harvest.

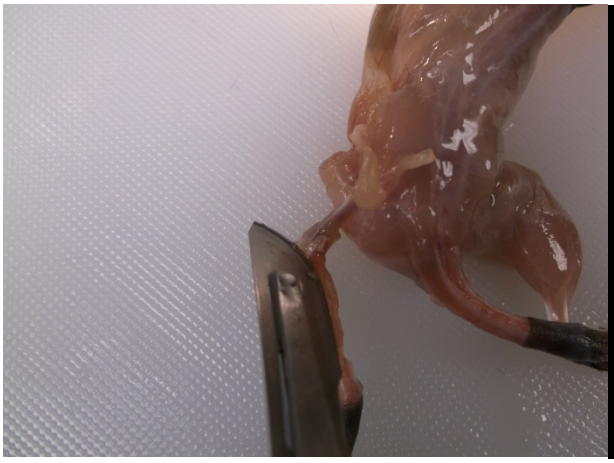
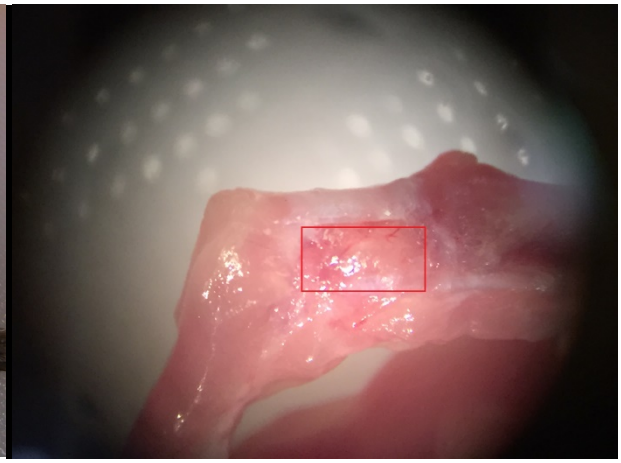



5) Using small scissors cut the upper leg muscles distally to proximally staying parallel and as close as possible to the femur. Several cuts may be needed. Then the muscles can be completely freed from the joint. Any small bits of muscle left over can be removed by scraping with a scalpel and/or tweezers.

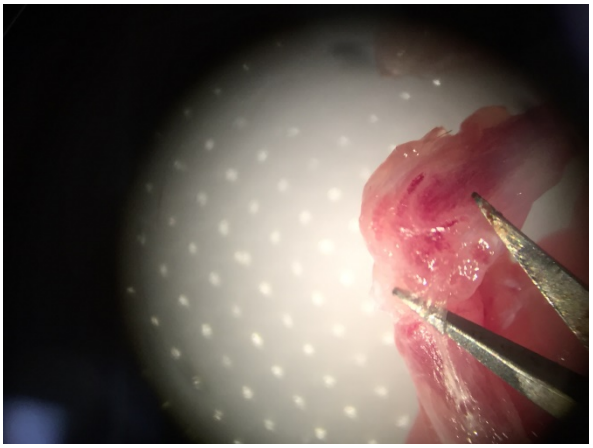
6) Using a similar method as step 5, free the gastrocnemius and other lower leg muscles, resulting in a joint free of major musculature.



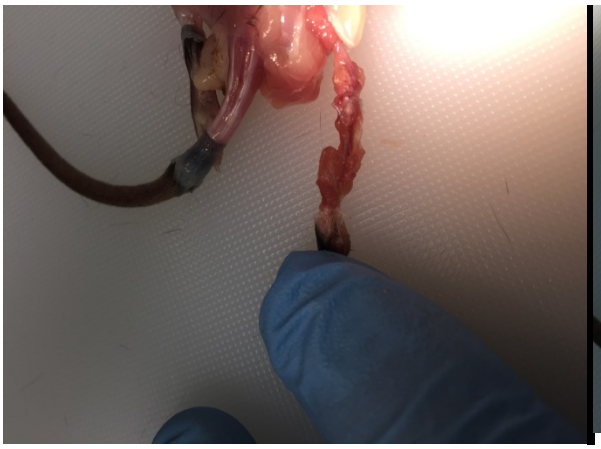
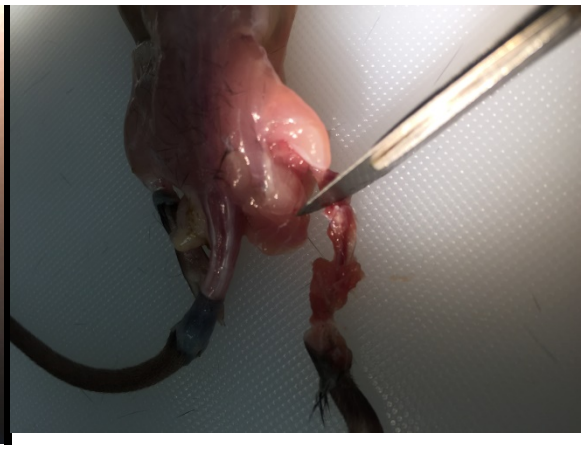
7) Harvest a 2mm X 4mm synovium sample from the lateral side of the joint using a scalpel to cut a box around the tissue being harvested (red lines), and the using forceps and the scalpel to completely remove it from the joint. Immediately rinse in sterile phosphate buffered saline (PBS) and then placed in a 24 well plate in 400um culture media

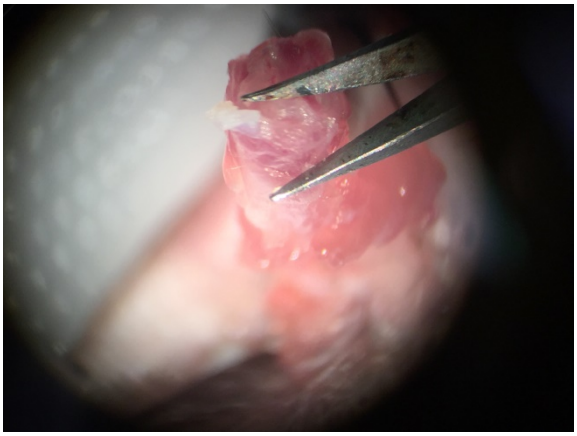
8) Severe all external connective tissue (LCL, MCL, Patellar tendon, Hamstring tendons) using scissors.



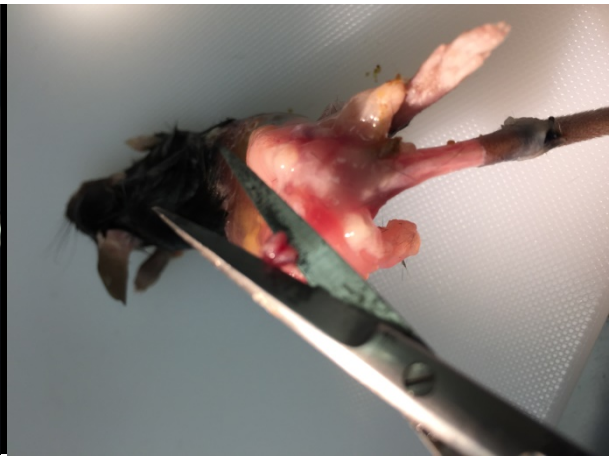
9) Using traction inferior to the joint to open up the joint space, separate using a sharp scalpel cut through the ACL and PCL.

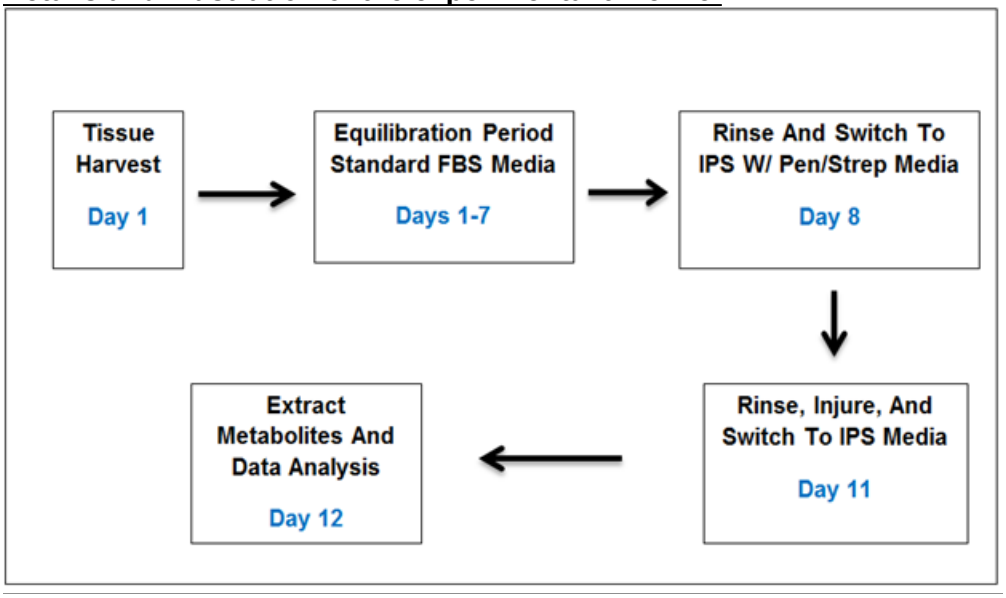
10) Carefully remove menisci from the tibial plateau using forceps and small scissors. Then rinse in sterile PBS and place in culture media in the 24 well plate.



11) Finally, remove both the tibial plateau and the femoral condyles from the long bones. This is facilitated by using sharp small scissors and ensuring to cut on the joint side of the epiphyseal plates. Wash in PBS and place into media in the 24 well plate.



Supplemental Figure 2.
Details and illustration of the experimental timeline.



References-

Anderson DD, Chubinskaya S, Guilak F, Martin JA, Oegema TR, Olson SA, Buckwalter JA. Post-traumatic osteoarthritis: improved understanding and opportunities for early intervention. J Orthop Res. 2011 Jun;29(6):802-9. doi: 10.1002/jor.21359. Epub 2011 Feb 11. Review. PubMed PMID: 21520254; PubMed Central PMCID: PMC3082940.

Andersson-Molina H, Karlsson H, Rockborn P. Arthroscopic partial and total meniscectomy: A long-term follow-up study with matched controls. Arthroscopy. 2002 Feb;18(2):183-9. PubMed PMID: 11830813.

Aoyama T, Liang B, Okamoto T, Matsusaki T, Nishijo K, Ishibe T, Yasura K, Nagayama S, Nakayama T, Nakamura T, Toguchida J. PGE2 signal through EP2 promotes the growth of articular chondrocytes. J Bone Miner Res. 2005 Mar;20(3):377-89. Epub 2004 Nov 29. PubMed PMID: 15746982.

Binfield PM, Maffulli N, King JB. Patterns of meniscal tears associated with anterior cruciate ligament lesions in athletes. Injury. 1993 Sep;24(8):557-61. PubMed PMID: 8244553.

Gupta T, Zielinska B, McHenry J, Kadmiel M, Haut Donahue TL. IL-1 and iNOS gene expression and NO synthesis in the superior region of meniscal explants are dependent on the magnitude of compressive strains. Osteoarthritis Cartilage. 2008 Oct;16(10):1213-9. doi: 10.1016/j.joca.2008.02.019. Epub 2008 Apr 24. PubMed PMID: 18439846.

Hall M, Wrigley TV, Metcalf BR, Cicuttini FM, Wang Y, Hinman RS, Dempsey AR, Mills PM, Lloyd DG, Bennell KL. Do Moments and Strength Predict Cartilage Changes after Partial Meniscectomy? Med Sci Sports Exerc. 2015 Aug;47(8):1549-56. doi: 10.1249/MSS.0000000000000575. PubMed PMID: 25397607.

Hennerbichler A, Fermor B, Hennerbichler D, Weinberg JB, Guilak F. Regional differences in prostaglandin E2 and nitric oxide production in the knee meniscus in response to dynamic compression. Biochem Biophys Res Commun. 2007 Jul 13;358(4):1047-53. Epub 2007 May 14. PubMed PMID: 17517372; PubMed Central PMCID: PMC2258009.

Jutila AA, Zignego DL, Hwang BK, Hilmer JK, Hamerly T, Minor CA, Walk ST, June RK. Candidate mediators of chondrocyte mechanotransduction via targeted and untargeted metabolomic measurements. Arch Biochem Biophys. 2014 Mar 1;545:116-23. doi: 10.1016/j.abb.2014.01.011. Epub 2014 Jan 16. PubMed PMID: 24440608; PubMed Central PMCID: PMC3967962.

Kamburov A, Cavill R, Ebbels TM, Herwig R, Keun HC. Integrated pathway-level analysis of transcriptomics and metabolomics data with IMPaLA. Bioinformatics. 2011 Oct 15;27(20):2917-8. doi: 10.1093/bioinformatics/btr499. Epub 2011 Sep 4. PubMed PMID: 21893519.

Katz JN, Solomon DH, Schaffer JL, Horsky J, Burdick E, Bates DW. Outcomes of care and resource utilization among patients with knee or shoulder disorders treated by general internists, rheumatologists, or orthopedic surgeons. Am J Med. 2000 Jan;108(1):28-35. PubMed PMID: 11059438.

McNulty AL, Guilak F. Mechanobiology of the meniscus. J Biomech. 2015 Jun 1;48(8):1469-78. doi: 10.1016/j.jbiomech.2015.02.008. Epub 2015 Feb 9. Review. PubMed PMID: 25731738; PubMed Central PMCID: PMC4442061.

Otsuka S, Aoyama T, Furu M, Ito K, Jin Y, Nasu A, Fukiage K, Kohno Y, Maruyama T, Kanaji T, Nishiura A, Sugihara H, Fujimura S, Otsuka T, Nakamura T, Toguchida J. PGE2 signal via EP2 receptors evoked by a selective agonist enhances regeneration of injured articular cartilage. Osteoarthritis Cartilage. 2009 Apr;17(4):529-38. doi: 10.1016/j.joca.2008.09.003. Epub 2008 Oct 14. PubMed PMID: 18922704.

Patti GJ, Yanes O, Siuzdak G. Innovation: Metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol. 2012 Mar 22;13(4):263-9. doi: 10.1038/nrm3314. Review. PubMed PMID: 22436749; PubMed Central PMCID: PMC3682684.

Petursson F, Husa M, June R, Lotz M, Terkeltaub R, Liu-Bryan R. Linked decreases in liver kinase B1 and AMP-activated protein kinase activity modulate matrix catabolic responses to biomechanical injury in chondrocytes. Arthritis Res Ther. 2013 Jul 25;15(4):R77. doi: 10.1186/ar4254. PubMed PMID: 23883619; PubMed Central PMCID: PMC3979085.

Praemer A, Furner S, Rice DP. Musculoskeletal Conditions in the United States. Rosemont, Ill: American Academy of Orthopedic Surgeons; 1999.

Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. Arterioscler Thromb Vasc Biol. 2011 May;31(5):986-1000. doi: 10.1161/ATVBAHA.110.207449. Review. PubMed PMID: 21508345; PubMed Central PMCID: PMC3081099.

Riera KM, Rothfusz NE, Wilusz RE, Weinberg JB, Guilak F, McNulty AL. Interleukin-1, tumor necrosis factor-alpha, and transforming growth factor-beta 1 and integrative meniscal repair: influences on meniscal cell proliferation and migration. Arthritis Res Ther. 2011;13(6):R187. doi: 10.1186/ar3515. Epub 2011 Nov 16. PubMed PMID: 22087734; PubMed Central PMCID: PMC3334636.

Scott GA, Jolly BL, Henning CE. Combined posterior incision and arthroscopic intra-articular repair of the meniscus. An examination of factors affecting healing. J Bone Joint Surg Am. 1986 Jul;68(6):847-61. PubMed PMID: 3755440.

Verdonk PC, Forsyth RG, Wang J, Almqvist KF, Verdonk R, Veys EM, Verbruggen G. Characterisation of human knee meniscus cell phenotype. Osteoarthritis Cartilage. 2005 Jul;13(7):548-60. PubMed PMID: 15979007.

Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, Djoumbou Y, Mandal R, Aziat F, Dong E, Bouatra S, Sinelnikov I, Arndt D, Xia J, Liu P, Yallou F, Bjorndahl T, Perez-Pineiro R, Eisner R, Allen F, Neveu V, Greiner R, Scalbert A. HMDB 3.0--The Human Metabolome Database in 2013. Nucleic Acids Res. 2013 Jan;41(Database issue):D801-7. doi: 10.1093/nar/gks1065. Epub 2012 Nov 17. PubMed PMID: 23161693; PubMed Central PMCID: PMC3531200.

Zhu ZJ, Schultz AW, Wang J, Johnson CH, Yannone SM, Patti GJ, Siuzdak G. Liquid chromatography quadrupole time-of-flight mass spectrometry characterization of metabolites guided by the METLIN database. Nat Protoc. 2013 Mar;8(3):451-60. doi: 10.1038/nprot.2013.004. Epub 2013 Feb 7. PubMed PMID: 23391889; PubMed Central PMCID: PMC3666335.

Zielinska B, Killian M, Kadmiel M, Nelsen M, Haut Donahue TL. Meniscal tissue explants response depends on level of dynamic compressive strain. Osteoarthritis Cartilage. 2009 Jun;17(6):754-60. doi: 10.1016/j.joca.2008.11.018. Epub 2008 Dec 13. PubMed PMID: 19121588.

Zignego DL, Hilmer JK, June RK. Mechanotransduction in primary human osteoarthritic chondrocytes is mediated by metabolism of energy, lipids, and amino acids. J Biomech. 2015 Dec 16;48(16):4253-61. doi: 10.1016/j.jbiomech.2015.10.038. Epub 2015 Oct 31. PubMed PMID: 26573901.