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Original Article

Articular cartilage and synovium may be important sources of post-surgical synovial fluid inflammatory mediators

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Abstract: The primary source of synovial fluid inflammatory mediators is currently unknown and may include different tissues comprising the joint, including the synovium and articular cartilage. Prior work in a porcine model has demonstrated that anterior cruciate ligament (ACL) surgery leads to significant changes in early gene expression in the synovium and articular cartilage, which are the same whether concomitant ligament restoration is performed or not. In this study, 36 Yucatan minipigs underwent ACL surgery, and a custom multiplex assay was used to measure synovial fluid protein levels of MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-12, MMP-13, IL-1α, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, GM-CSF, and TNFα in 18 animals at 1 and 4 weeks after surgery. Linear regressions were used to evaluate the relationships between synovial fluid protein levels and the previously reported gene expression levels in the articular cartilage and synovium from the same animal cohort. Synovial fluid levels of MMP-13 and IL-6 were significantly correlated with synovial gene expression (P=.003 and P<.001 respectively), while IL-1α levels were significantly correlated with articular cartilage gene expression (P=.037). The synovium may be an important source of MMP-13 and IL-6, and the articular cartilage may be an important source of IL-1α in post-surgical inflammation. In developing treatments for post-surgical inflammation, the synovium may therefore be a promising target for modulating inflammatory mediators such as MMP-13 and IL-6 in the synovial fluid.

Keywords: Inflammation, osteoarthritis-post traumatic, synovium, cartilage, knee, biomarkers, animal model

Introduction

Post-surgical or post-injury joint inflammation has been implicated in the development of prolonged post-operative pain [1], arthrofibrosis [2], and osteoarthritis [3], with the interleukins and matrix metalloproteinases found in the synovial fluid of inflamed joints thought to play key roles in these manifestations [4]. While inflammation has long been thought to play an essential role in the post-operative course, the primary tissue sources for the different inflammatory cytokines and matrix metalloproteinases (MMPs) remain to be elucidated.

Within the first four weeks after transection of the anterior cruciate ligament (ACL) in the porcine model, a post-surgical inflammatory response has been noted, consisting of changes in the histology of the synovium and cartilage, as well as in the gene expression of the two tissues, regardless of whether a concomitant ACL restoration or reconstruction procedure was performed [5, 6]. Prior studies have reported an increase in the concentrations of inflammatory cytokines and MMPs in the synovial fluid following joint injury, particularly for IL-1Ra, IL-4, IL-6, IL-12, and IL-18 [7-14]. However, the primary tissue sources for these inflammatory mediators are unknown. Possibilities include secretion by the articular cartilage, synovium, another intra-articular tissue, or a transudate of serum. We elected to begin to explore this question by looking for correlations between the gene expression of these mediators, as previously determined using

Table 1. Genes encoding the seven matrix metalloproteinases (MMPs) and eleven cytokines included in this analysis

MMPs	Cytokines
MMP-1	*IL-1A*
MMP-2	*IL-2*
MMP-3	*IL-4*
MMP-7	*IL-6*
MMP-9	*CXCL8*
MMP-12	*IL-10*
MMP-13	*IL-12A*
	IL-12B
	IL-18
	CSF2
	TNF

RNA-Seq [5, 6], with newly acquired synovial fluid protein level data to determine if either the articular cartilage or synovium were reasonable candidates as the contributors. As there were no differences in gene expression or histology among the surgical groups, and no macroscopic cartilage damage in any group at the 1- or 4-week time point, the data from the three surgical groups and two time points were pooled to improve our ability to detect significant correlations between the inflammatory cytokines or MMPs and the tissue RNA expression of the genes for those proteins. We selected 11 cytokines and 7 MMPs previously associated with joint inflammation [4, 15-18], of which we also had gene expression data from the articular cartilage and synovium [5, 6] (**Table 1**).

Our primary hypothesis was that protein levels for the molecules of interest, in the synovial fluid would significantly relate to gene expression in the articular cartilage, synovium, both, or neither, when all surgical groups and time points were combined. In addition, we hypothesized that the gene expression of the cytokines and MMPs of interest in the articular cartilage would correlate with the gene expression of the same proteins in the synovium.

Materials and methods

Study design

A controlled, large animal experiment with cross-sectional outcome assessments at two post-surgery time points was designed. The Institutional Animal Care and Use Committee approved this study (Brown University #1511-000175), which was performed in accord with ARRIVE guidelines [19]. Thirty-six adolescent Yucatan minipigs (Sinclair BioResources, Columbia MO) were allocated to receive unilateral ACL transection surgery (n=36) followed by euthanasia and outcome assessments at 1 week (1 W, n=18) or 4 weeks (4 W, n=18) after surgery. Within each time point, 6 of the 18 animals were allocated to no treatment following transection, 6 of the 18 to immediate ligament reconstruction surgery, and 6 of the 18 to immediate ligament restoration surgery (**Figure 1**). A computer-based random permutation stratified for sex determined each animals group allocation and side of unilateral surgery. The current analysis leverages the previously published RNA-Seq data of the synovium and articular cartilage from this same cohort [5, 6], and added the newly acquired data for synovial fluid protein levels for each animal obtained at the time of euthanasia, as well as the correlation analyses between the protein levels and RNA expression. Justification for the animal model, sample size, details for the IACUC approved surgical procedures, animal husbandry, and pain management have been previously reported [5, 6], and are provided in Supplementary Material 1.

Articular cartilage, synovium and synovial fluid sample collection

After euthanasia, synovial fluid was aspirated and centrifuged at 1300 relative centrifugal force (RCF), and the supernatant stored at -80°C in 50 ul aliquots until analysis. If the initial aspiration was unsuccessful, the collection was repeated after a 10 cc phosphate buffered saline injection, with a serum/synovial fluid urea concentration ratio used to calculate the dilution factor as previously described [20]. After fluid aspiration, the knee joints were opened using aseptic technique. Four to eight 5 mm diameter osteochondral samples were harvested for RNA isolation from the area posterior to the frontal plane in the center of the medial femoral condyle. Osteochondral samples were rinsed in saline and the cartilage was separated from the subchondral bone. Cartilage samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA

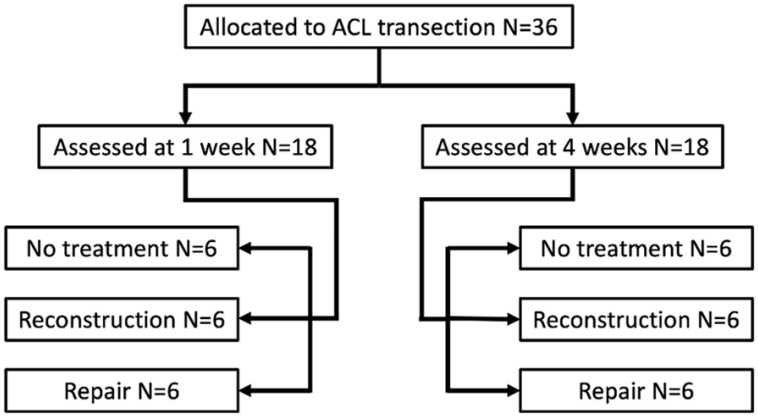

Figure 1. Consort diagram for the animal study that produced the tissue samples used in this analysis.

isolation. The synovium attached to the posterior half of the medial meniscus, remote from the prior anterior arthrotomy site, was harvested for immediate RNA extraction.

Articular cartilage RNA-Seq

Immediately following surgical excision, articular cartilage samples were frozen until homogenization. Homogenization procedures were performed as previously described [6]. After homogenization, samples were frozen again until RNA was extracted using phenol-chloroform separation, purified, treated with DNase, and assessed for purity and integrity. The samples were then enriched for poly(A+) messenger RNA, reverse transcribed, ligated, and amplified with 17 cycles of polymerase chain reaction using an Illumina TruSeq RNA Sample Preparation Kit Version 2, which involved the use of 50-bp paired-end reads. Reads were then aligned to the susScr3 genome (assembled by the Swine Genome Sequencing Consortium [21]) and accessed through a genome browser hosted by the University of California, Santa Cruz (https://genome.ucsc.edu/). Read alignment was handled by the RNA-Seq Unified Mapper (RUM) developed at the University of Pennsylvania [22]. Gene counts were then generated with a custom R script [6]. Counts were then supplied to DESeq2, which normalized counts to provide median of ratios [23]. DESeq2's median of ratios accounts for sequencing depth and composition of the RNA by dividing raw counts by size factors that are specific to each tissue sample. These size factors were calculated by taking the median ratio of the counts per gene relative to the geometric mean of each gene.

Synovium RNA-Seq

Unlike the articular cartilage samples, synovium samples underwent homogenization immediately following excision. RNA extraction and integrity assessment procedures are detailed in Sieker et al. (2018) [5]. The same library preparation and bioinformatics tools, mentioned above for the articular cartilage samples, were used to generate median of ratios for the synovium samples.

Synovial fluid multiplex assay

A custom multiplex assay kit (SPR#1178, Millipore, Burlington, MA) was used to assess the concentrations of our target proteins (**Table 1**). Synovial fluid samples were assayed by time point with all three treatment groups pooled together (**Figure 1**). Each synovial fluid sample had a duplicate run in the same batch.

Multiplex technology (Bioplex-200; BioRAD, Hercules, CA) was used to measure fluorescent intensity. Mean fluorescent intensities (MFIs) and concentrations of standards were used to establish a standard curve. MFIs served as inputs for 5PL logistic regressions to obtain concentration estimates. Concentration estimates were then averaged between duplicates using commercial software (Bioplex Manager; BioRAD, Hercules, CA). For each sample, the ratio of synovial fluid urea concentration (post-averaging) to serum urea concentration was obtained using a blood urea nitrogen (BUN) assay (ab83362, Cambridge, MA) [24]. Concentrations were expressed in picograms per milliliter (pg/ml).

It is important to note that synovial fluid protein levels were detected for IL-1Ra and MMP-10, but they were not included in this analysis because there are no orthologues annotated in the susScr3 genome (i.e., no RNA-Seq expression values can be calculated for the corresponding *IL-1RN* and *MMP-10* genes) [25].

Additionally, levels of the IL-12 protein were used for analyses with both the *IL-12A* as well as the *IL-12B* genes.

Statistical analysis

All statistical analyses, described below, were carried out in R version 4.0.1 [26]. Gene expression levels were obtained by converting raw counts to median of ratios as described above; there were no adjustments for lower or upper bounds before including these values in the analysis. Protein levels, however, underwent lower bound replacement for synovial fluid samples that were deemed below the lower detectable limit of the multiplex assay. Specifically, samples with readings below the lower bound were replaced by zero for each respective cytokine or MMP.

All treatment groups and both time points were pooled. Linear regressions were used to evaluate the relationship between synovial fluid protein levels and gene expression levels in different tissues. Significant regressions are reported in the main text, and all regressions are reported in Supplementary Material 2. Spearman correlations were also performed on all regression datasets with significant results. This analysis served to bolster relationship findings by providing an assessment that was both non-parametric and more satisfactory in meeting its assumptions (i.e., that the data could be modeled by a monotonic function). Pearson correlations were performed on expression data (median of ratios) between articular cartilage and synovium for each cytokine and MMP of interest. Significant results are reported in the main text, and all correlation results are provided in Table S1; Figures S1, S2, S3, S4. Pearson correlation plots and regression plots both feature 95% confidence intervals.

We assessed regression models for outliers by calculating studentized residuals-values for each sample that were determined by dividing regular residuals by a term that includes the average and the mean square error with the sample of interest removed (https://online.stat.psu.edu/stat462/node/247/). Samples with studentized residuals above 3.0 (absolute value) were deemed outliers. This exclusion resulted in one reconstruction animal at one week being excluded from the *MMP-1* analyses, one restoration subject at one week being excluded from the *MMP-3*, *MMP-12,* and *MMP-13* analyses, and a second restoration subject at one week being excluded from the *MMP-12* synovium analyses. Similarly, the outlier exclusion criteria led to one restoration animal at one week being excluded from the *IL-1A*, *IL-4*, and *IL-12B* analyses. These removed outliers are visualized in Figures S3 and S4.

A *P*-value of 0.05 served as the threshold for significance, with mentioning of relationships that fell slightly short of this metric. We did not adjust *P*-values for multiple comparisons. This was done to decrease the type II error rate and increase the discovery of "true positives" (in exchange for possibly increasing the type I error rate).

Results

Demographic data for the 36 animals used in this analysis are presented in Table S1. There were no significant differences in baseline age, baseline weight, and sex distribution between all groups as previously reported [6]. No adverse events were observed during surgery or follow-up.

Most cytokine and MMP analyses excluded at least one animal due to undetectable multiplex values, but the average age of subjects varied by less than eight days between groups, and the average weight varied by less than 0.6 kg between groups. The percentage of animals with synovial fluid levels above the lower detectable limit is shown in **Table 2**.

MMPs were expressed in almost all samples of articular cartilage and synovium obtained from the joints post-injury (**Table 3**). For the cytokines, *IL-10* and *IL-18* were present in almost all the samples of articular cartilage and synovium (**Table 4**), while *IL-2*, *IL-4,* and *CXCL8* had poor coverage in both the cartilage and synovium. *IL-1A* and *TNF* were present in approximately half of the cartilage samples, and in all the synovium samples. Lastly, while *IL-12B* and *CSF2* were present in a low percentage of cartilage samples, they were each present in approximately half of the synovium samples (**Table 4**).

Regression of synovial fluid protein levels and gene expression in the cartilage and synovium

Matrix metalloproteinase analyses: MMP-13 (P=.003) had a significant relationship between

Table 2. Percentage of samples with synovial fluid protein levels above the lower detectable limit

Protein	% Above Detectable Limit
MMP-1	100
MMP-2	13
MMP-3	57
MMP-7	14
MMP-9	58
MMP-12	17
MMP-13	77
IL-1α	46
IL-2	52
IL-4	56
IL-6	94
IL-8	61
IL-10	49
IL-12	72
IL-18	100
GM-CSF	29
TNFα	3

Table 3. Percentage of samples with non-zero mRNA counts for MMPs in the articular cartilage and synovium

Gene	% Synovium	% Cartilage
MMP-1	100	100
MMP-2	100	100
MMP-3	100	100
MMP-7	94	81
MMP-9	100	100
MMP-12	92	61
MMP-13	100	100

synovial fluid protein levels and synovial membrane gene expression, and there was some evidence of similar relationships for MMP-1 (P=.075) and MMP-12 (P=.09). There was also some evidence of a relationship between synovial fluid levels of MMP-3 and articular cartilage gene expression of *MMP-3* (P=.065). **Figure 2** depicts these regressions, with the MMP-12 model removed due to heteroskedasticity. Regressions for all MMPs can be found in Supplementary Material 2.

Cytokine analyses: Synovial fluid levels of IL-1α were significantly related to *IL-1A* expression in articular cartilage (P=.037) (**Figure 3**). Synovial fluid levels of IL-6 and GM-CSF were significantly related to synovium expression of *IL-6* (P<.001) and *CSF2* (P=.016) respectively, and there was some evidence of relationships between synovial fluid levels of IL-4 and IL-12 and synovium expression of *IL-4* (P=.066) and *IL-12B* (P=.083) (**Figure 3**). Models for IL-4 and GM-CSF were removed due to heteroskedasticity. Regressions for all other cytokines can be found in Supplementary Material 2.

Table 4. Percentage of samples with non-zero mRNA counts for the cytokines of interest in the articular cartilage and synovium

Gene	% Synovium	% Cartilage
IL-1A	100	51
IL-2	9	0
IL-4	13	3
IL-6	47	6
CXCL8	9	0
IL-10	100	86
IL-12A	61	28
IL-12B	53	8
IL-18	100	100
CSF2	54	14
TNF	100	60

Correlation of gene expression in articular cartilage and gene expression in synovium

Gene expression in the articular cartilage had a significant correlation with gene expression in the synovium for *MMP-1* (R=.35, P=.035) and *MMP-3* (R=.39, P=.019) (**Figure 4**). Gene expression in articular cartilage had a significant correlation with gene expression in synovium for *TNF* (R=.37, P=.029), and there was some evidence of a similar correlation for *IL-1A* (R=.33, P=.052) (**Figure 5**). Correlation plots for all MMPs are in Figure S1, and correlation plots for all cytokines are in Figure S2.

Discussion

In this study, we found that synovium gene expressions of *MMP-13* and *IL-6* were significantly related to levels of associated proteins in the synovial fluid, with reasonable evidence of similar relationships for *MMP-1*, *MMP-12* and *IL-12B*. The articular cartilage gene expression of *IL-1A* was significantly related to synovial fluid levels of IL-1α, and there was reasonable evidence of a similar relationship for *MMP-3*. These findings suggest that the synovi-

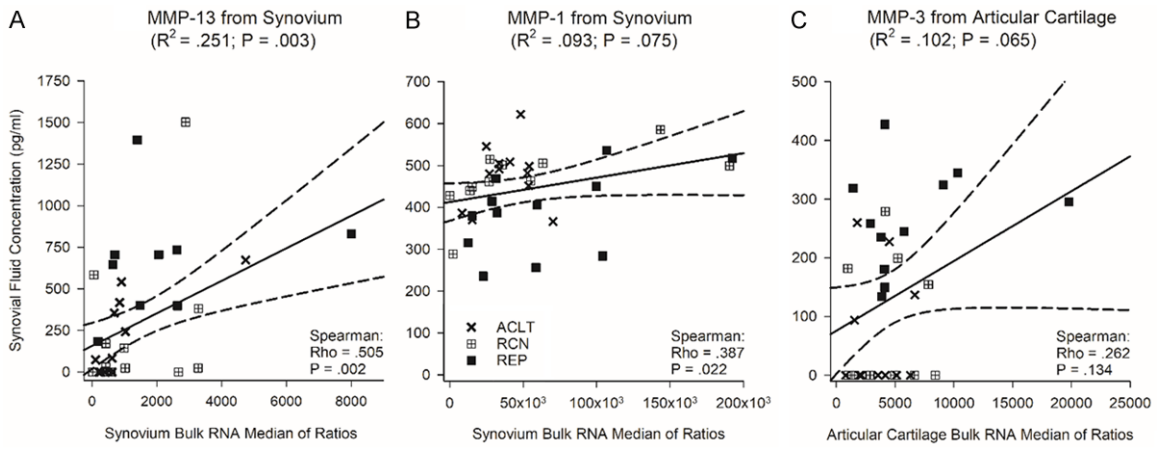

Figure 2. A. Of the seven MMPs evaluated, MMP-13 featured a significant relationship between synovial fluid protein levels and gene expression in the synovial membrane. B. Similarly, MMP-1 also featured some evidence of a relationship between synovial fluid and gene expression in the synovium. C. MMP-3 had some evidence of a relationship between the protein level in the synovial fluid and the gene expression in the articular cartilage. ACLT=ACL transection, RCN=ACL reconstruction, REP=ACL restoration. The regression line (solid) and 95% confidence intervals (dashed) for the groups pooled are also provided.

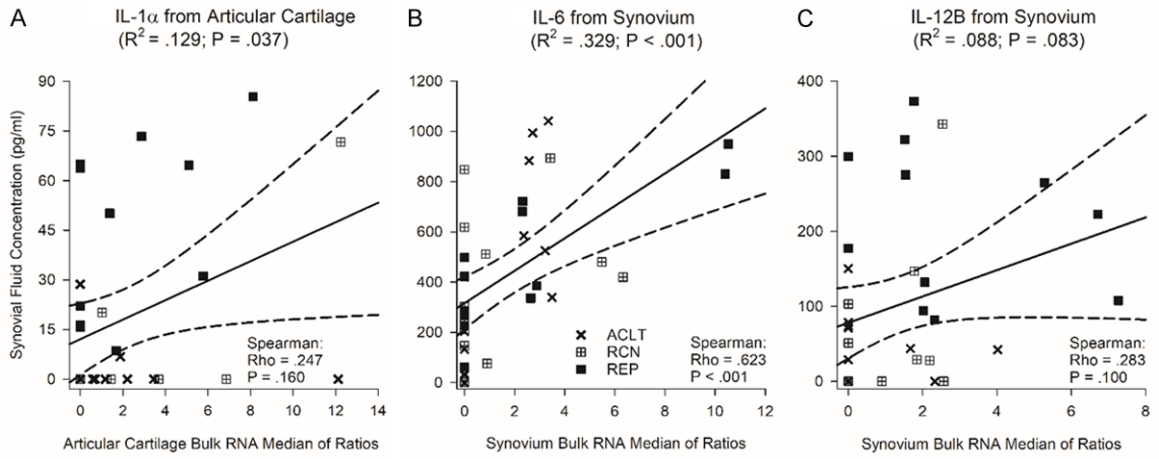

Figure 3. A. There was a significant relationship between the IL-1α protein level in the synovial fluid and *IL-1A* expression in articular cartilage. B. There was a significant relationship between IL-6 protein in synovial fluid and IL-6 expression in synovium. C. There was some evidence of a similar relationship between IL-12 protein in synovial fluid and *IL-12B* expression in synovium. While GM-CSF appeared to have a statistically significant relationship, it was removed due to heteroskedasticity. ACLT=ACL transection, RCN=ACL reconstruction, REP=ACL restoration. The regression line (solid) and 95% confidence intervals (dashed) for the groups pooled are also provided.

um may be an important source of MMP-13 and IL-6 in the synovial fluid during post-surgical inflammation, while the articular cartilage may be an important source of IL-1α.

The current association of mRNA expression of *MMP-13* and the level of the protein in the synovial fluid is consistent with prior reports of significant expression of *MMP-13* in the synovium from patients with rheumatoid arthritis [27]. To date, much of the work on *MMP-13* and osteoarthritis has focused on the production of *MMP-13* within the articular cartilage, and in blocking the production of this enzyme in the articular cartilage [28-30]. However, these studies did not evaluate the mRNA expression for *MMP-13* in the synovium, nor did they establish a statistical connection between the mRNA expression in either tissue with the level in the synovial fluid. One study, however, did verify the presence of MMP-13 in human synovium using immunohistochemistry, but

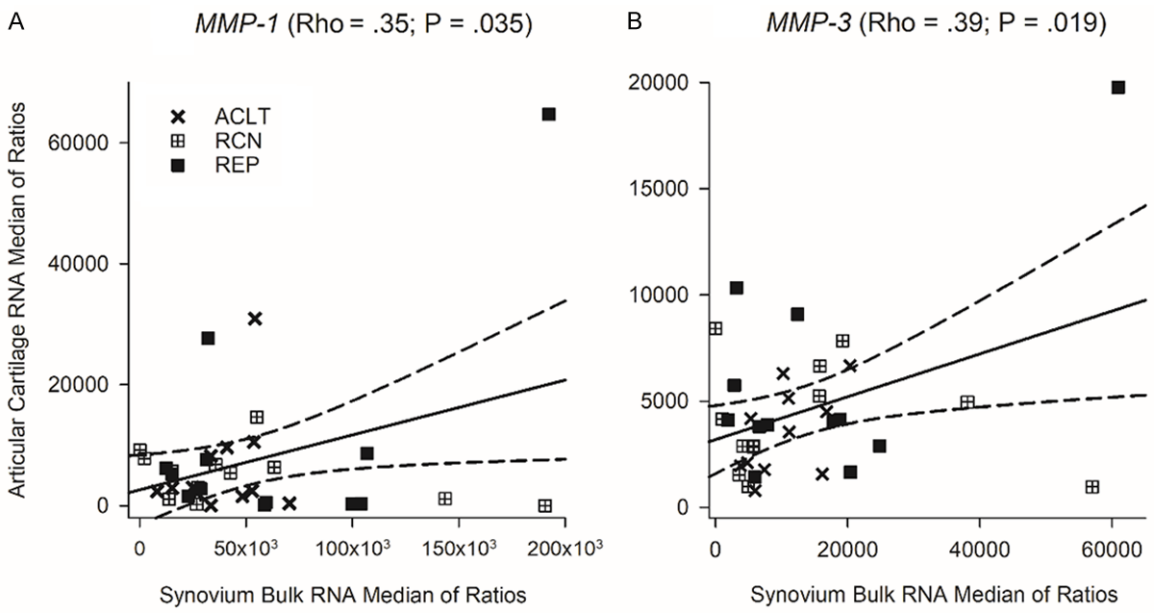

Figure 4. Of the seven MMPs evaluated, there were significant Pearson correlations between the mRNA expression in the articular cartilage and in the synovial membrane for (A) *MMP-1* and (B) *MMP-3*. ACLT=ACL transection, RCN=ACL reconstruction, REP=ACL restoration.

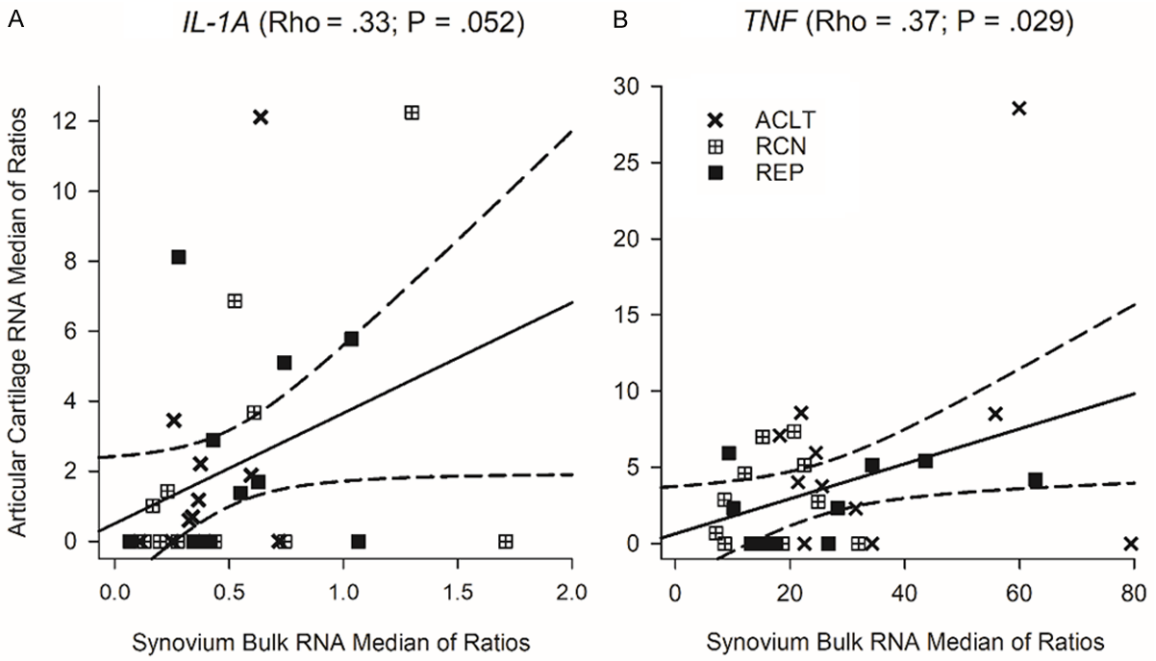

Figure 5. Of the eleven cytokines evaluated. B. *TNF* featured a significant Pearson correlation between mRNA expression in articular cartilage and the synovial membrane. A. *IL-1A* had similar results, though not significant. ACLT=ACL transection, RCN=ACL reconstruction, REP=ACL restoration.

samples were derived from a different disease context-late-stage osteoarthritis and rheumatoid arthritis [31]. Our results here suggest that blocking *MMP-13* production within the synovium in the early stages of inflammation may be useful for limiting the exposure of the superficial cartilage to this enzyme.

While prior studies have reported increased synovial fluid protein levels of IL-6 in disease

states associated with synovial inflammation (e.g., rheumatoid arthritis, osteoarthritis, obesity) [32-34], they neither simultaneously sequenced the synovium nor restricted their analyses to the early-stage disease setting; thus failing to introduce the hypothesis that the synovium could be the primary source of IL-6. One study, however, did evaluate the presence of IL-6 in synovium using immunohistochemistry and RT-PCR, but their IL-6 analysis was restricted to chronic autoimmune arthritis in non-knee murine joints [35]. Our study addressed the IL-6 synovium hypothesis in a post-surgical inflammation model, where direct sequencing of the synovium resulted in a significant correlation between synovial gene expression of *IL-6* and the synovial fluid protein level of IL-6. This finding suggests that the synovium may be an important source of IL-6 in post-surgical joints.

The origin of synovial fluid protein levels of IL-1α in the osteoarthritic process remains unresolved. *In vitro* studies of chondrocytes, wherein the cells were dissociated from their matrix and passaged, failed to detect *IL-1A* gene expression [36]. In contrast, the presence of *IL-1A* mRNA in normal chondrocytes [37], and increased immunohistochemistry staining for IL-1α have been demonstrated in human cartilage and canine cartilage, respectively [38, 39]. In addition, the intra-articular administration of IL-1Ra, a ligand that binds to surface receptors on IL-1a and renders it non-inflammatory, has led to improved cartilage status in canine and rat models [40-43]. However, a clinical study aimed at directly blocking IL-1α with subcutaneous administration of an anti-IL-1α/β drug did not demonstrate efficacy in reducing symptoms of osteoarthritis [44]. However, this clinical study did not verify whether IL-1α was effectively lowered in the joint environment with the administered subcutaneous treatment [44]. The current porcine study adds to the discussion in that a significant relationship between articular cartilage *IL-1A* expression and IL-1α protein levels in synovial fluid was noted.

In addition to serving as a window into the molecular dynamics of post-surgical inflammation, the current study also characterized the relationships between the knee joint tissues following ACL transection. Although the findings are too preliminary to solidify a mechanistic understanding, they suggest that ACL disruption engenders relationships between synovium and synovial fluid-mediated by MMP-13 and IL-6 expression-as well as between articular cartilage and synovial fluid-mediated by IL-1α. These signaling molecules have historically been implicated in the pathogenesis of cartilage destruction in osteoarthritis and rheumatoid arthritis [45], and the current study offers insight into which tissues are introducing them into the joint environment early on after surgery. However, the direct stimuli, which induce synovium and cartilage expression of these molecules, remain to be seen. Possibilities include direct tissue crosstalk between the torn ligament and surrounding structures, altered loading which results from joint destabilization following ligament disruption, or both.

The current study has a number of limitations that warrant consideration. Pigs are quadrupeds; thus, the mechanics and post-surgical inflammation may differ from human manifestations of the disease. However, the porcine model has been shown to exhibit knee joint biomechanics and osteoarthritis progression after injury [46, 47], which are similar to those seen in humans [48, 49]. In addition, this was a study of adolescent animals, and thus precludes extension of these findings to juvenile or adult animals. Furthermore, only cartilage and synovial RNA expression were studied here, and we could only provide evidence for cytokines and matrix metalloproteinases from those two tissues. Future studies to evaluate other intra-articular structures, including ligament and menisci, are planned. This study only evaluated the changes that occurred within the first four weeks of the surgical insults. Future studies are needed to evaluate the long-term relationships between gene expression and synovial fluid profiles. Moreover, the current study did not employ immunohistochemistry or flow cytometry, which could have served to confirm the origin of the molecules of interest. Following up on the current findings using these techniques would be a promising future step. Another limitation is that in our calculations, the multiplex protein levels below the lower detectable limit were replaced with values of zero for the regression and correlation tests, an accommodation that assumes these subthreshold values are biologically irrelevant.

This study also has unique strengths. It represents one of the only *in vivo* large animal studies that characterizes synovial fluid protein contents in the context of tissue transcript expression. Previous preclinical osteoarthritis studies have evaluated joint presence of IL-1α and MMP-13, but they did not evaluate how tissue expression relates to synovial fluid protein content [38, 41-43, 50]. Moreover, the evaluation of both synovium and articular cartilage makes this study a unique synthesis of multiple transcriptomic comparisons to synovial fluid composition. The current study also reaffirms the presence of certain pro-inflammatory molecules in the synovial fluid and joint tissues and their likely contribution to the post-surgical inflammation.

In conclusion, this study highlights the relationships between the expression of MMP and interleukin genes in knee joint tissues and their protein levels in synovial fluid in post-operative inflammation-comparisons that suggest the synovium as an important source for MMP-13 and IL-6 and cartilage as an important source of IL-1α during this early period. Future studies evaluating the intra-articular communication between joint tissues and synovial fluid in acute and chronic inflammation are warranted.

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Disclosure of conflict of interest

Dr. Murray is a founder and equity holder, Dr. Proffen is a paid consultant and equity holder, and Dr. Fleming is a founder of Miach Orthopaedics, Inc., which was formed to upscale production of a scaffold for ACL restoration and is related to one of the procedures described herein. Drs. Murray and Proffen maintain a conflict-of-interest management plan approved by Boston Children's Hospital while Dr. Fleming maintains a similar plan with Rhode Island Hospital.

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Supplementary Material 1: Supplemental Methods

Animal model

The adolescent Yucatan minipig model was selected for this study as it exhibits various features of human knee joints [1]. The Yucatan minipig has been shown to develop macroscopic cartilage lesions consistent with posttraumatic osteoarthritis (PTOA) within one year following ACL transection [2]. The cartilage damage typically develops in the medial compartment with more pronounced damage at areas adjacent to the tibial spine [2], consistent with the damage observed in human patients following ACL reconstruction surgery [3]. Furthermore, the Yucatan minipig model has also been shown to develop other non-cartilaginous features of PTOA, such as an early synovitis along with accompanying changes in protein markers of extracellular matrix breakdown [4]. The genetic [5] and pharmacokinetic [6] similarities between the porcine model and humans further support the use of the Yucatan minipig ACL transection model to study PTOA.

Housing and husbandry

Following delivery to the animal care facility, all animals underwent a minimum of a 7-day quarantine and stabilization period. The pigs were housed in single cages (a minimum of 22.5 ft^2) with wood chips over the concrete floor. All pigs were housed in pens that were adjacent to pens housing other pigs. Pigs were allowed to ambulate at all times. Animals were fed at several scheduled times per day. However, food was withheld a minimum of 12 hours before surgery and before euthanasia. No animals were excluded from the study and no modifications to the approved protocol were necessary over the course of the study. The animals were not used in any previous study and were considered healthy via veterinarian examination upon arrival and the joints determined to be normal via intraoperative assessment.

Anesthesia

Anesthesia was induced using Telazol (4 mg/Kg) and Xylazine (2 mg/kg) followed by Propofol (3-7 mg/kg) and then maintained with Isoflurane (1-3 MAC) following intubation. Eyes were protected using an eye lubricant. Both limbs were shaved and scrubbed with Chlorhexidine and 70% alcohol until visibly clean, followed by a ten-minute evaporation period. Hoofs were covered with unsterile gloves. Animals were then transferred into the adjacent operating room, placed supine on a heating mat, and secured on the operation table. Animal health and anesthesia depth were maintained by monitoring respiratory rate, oxygen saturation, electrocardiogram, blood pressure, and body temperature. The surgical limb and ipsilateral lower body were then scrubbed three times using Betadine. Hoofs were covered with a sterile glove and secured using a sterile elastic wrap. One layer of sterile towels was placed around the surgical area, followed by a layer of sterile drapes, leaving only the surgical limb exposed during the procedure.

Euthanasia

Prior to euthanasia, anesthesia was induced and maintained similar to that used for the surgical procedures. Animals were euthanized during deep anesthesia using an intravenous injection of a solution containing pentobarbital sodium and phenytoin sodium (Beuthanasia-D, 0.1 ml/kg). Death was confirmed by a veterinarian technician by the absence of blood pressure and heart sounds prior to obtaining the tissue samples.

Analgesia and peri-operative care

Drug	Dose		Route	Frequency of application (times/day)	Duration (days)
	mg/kg	ml			
Buprenorphine	0.01		Intramuscular	Once, pre-op	1
Fentanyl Patch	2 ug/kg/hr		Transdermal	Once, pre-op	3
Ceftiofur	5		Intramuscular	Once, pre-op	1
0.5% Bupivicaine + 2% Lidocaine		1.0	Subcutaneous, around wound	Once, pre-op	1
Ondansetron	4		Intramuscular or Intravenous	Once, pre-op	1
Tylenol elixir	10-15		Orally	Every 6 hours	As needed

Surgical procedures

ACL transection

A medial arthrotomy was created and the fat-pad partially resected to expose the ACL. The ACL was cut between the proximal and middle thirds of the ligament. A Lachman test was performed to verify complete transection. The knee was then irrigated with 500 cc of normal saline. For those animals assigned to receive no treatment, the incision was then closed in layers [2], and the ligament was allowed to heal naturally.

ACL reconstruction

Following ACL transection in the animals assigned to the ACL reconstruction group, fresh-frozen BPTB allografts, which were harvested from age, weight, and gender matched donors, were implnated as previously described [2]. The entire patellar tendon (~10 mm in width) was used for the soft tissue portion of the graft while the bone plugs were trimmed to 7 mm diameter. Femoral graft fixation was achieved with a 6×20 mm bio-absorbable interference screw (Biosure; Smith & Nephew, Andover, MA). The graft was manually pre-conditioned in tension twenty times and firmly tensioned with the knee in maximal extension (~30° for the pig). The distal block was secured in the tibia using a second 6 mm interference screw backed up with an extracortical tibial button. All incisions were closed in layers.

Bridge-enhanced ACL restoration

For the animals assigned to the bio-enhanced ACL restoration group, the repair was performed following ACL transection as previously described [2]. In brief, an Endobutton carrying three looped sutures was passed thru a 4 mm femoral tunnel and flipped. Two of the sutures were threaded through the scaffold, into a predrilled tibial tunnel and fixed extracortically using a button with the knee in maximum extension. The remaining suture was tied to a Kessler suture of #1 Vicryl (Ethicon, Somerville, NJ), which had been placed in the tibial stump of the ACL. Three cubic centimeters of autologous blood were used to saturate and activate the scaffold. The scaffold-blood composite was allowed to set for a minimum of 10 minutes before completion. All incisions were closed in layers.

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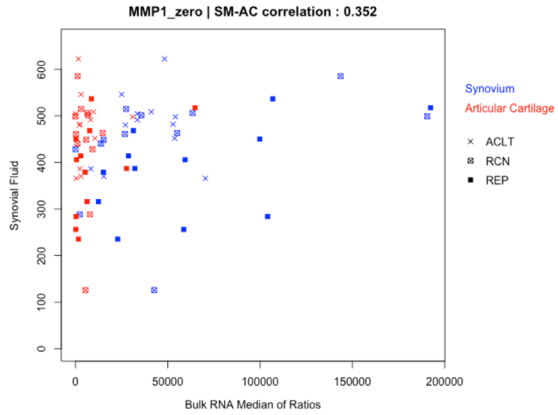
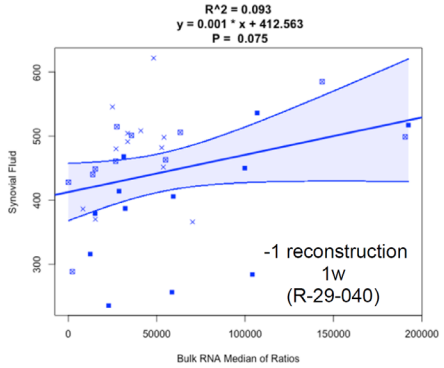
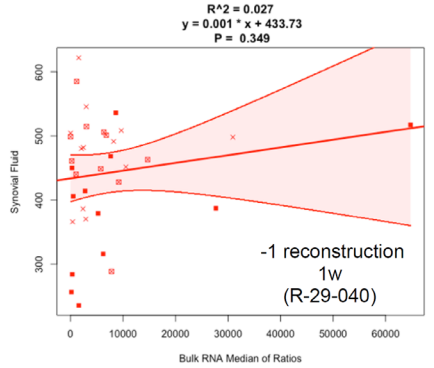
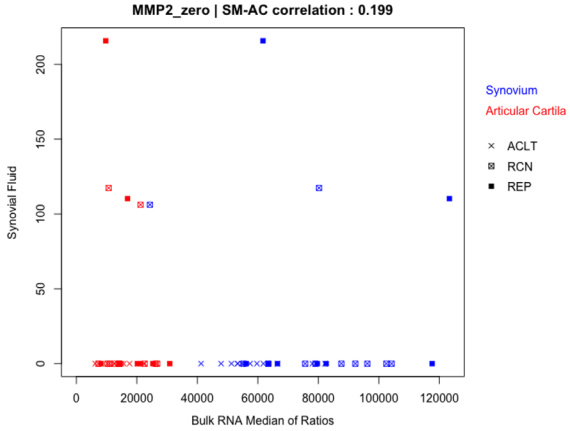
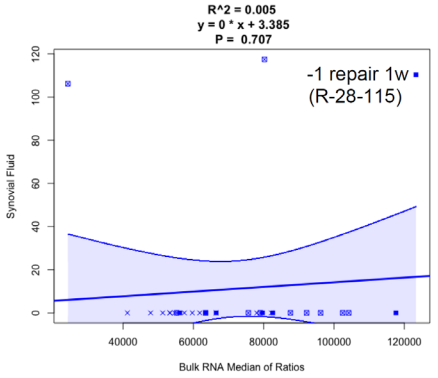
Gene and protein expression in the post-surgical joint

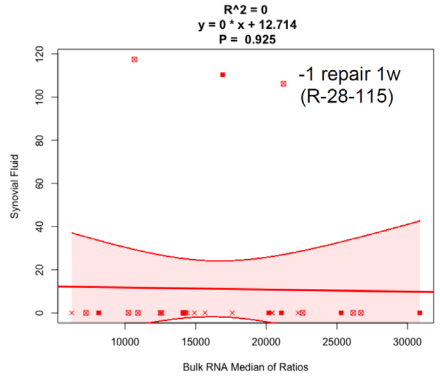


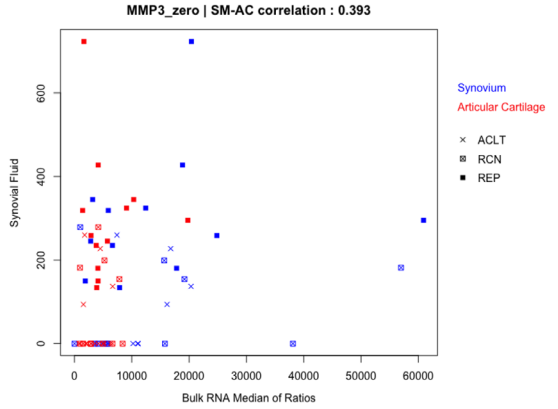


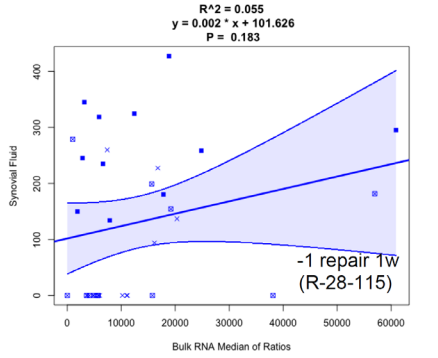


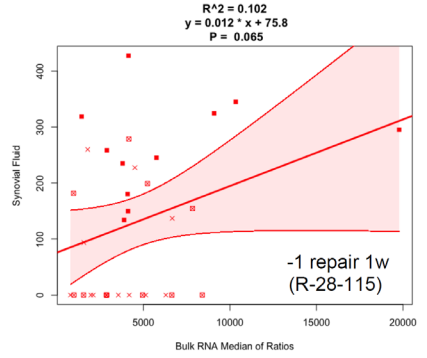




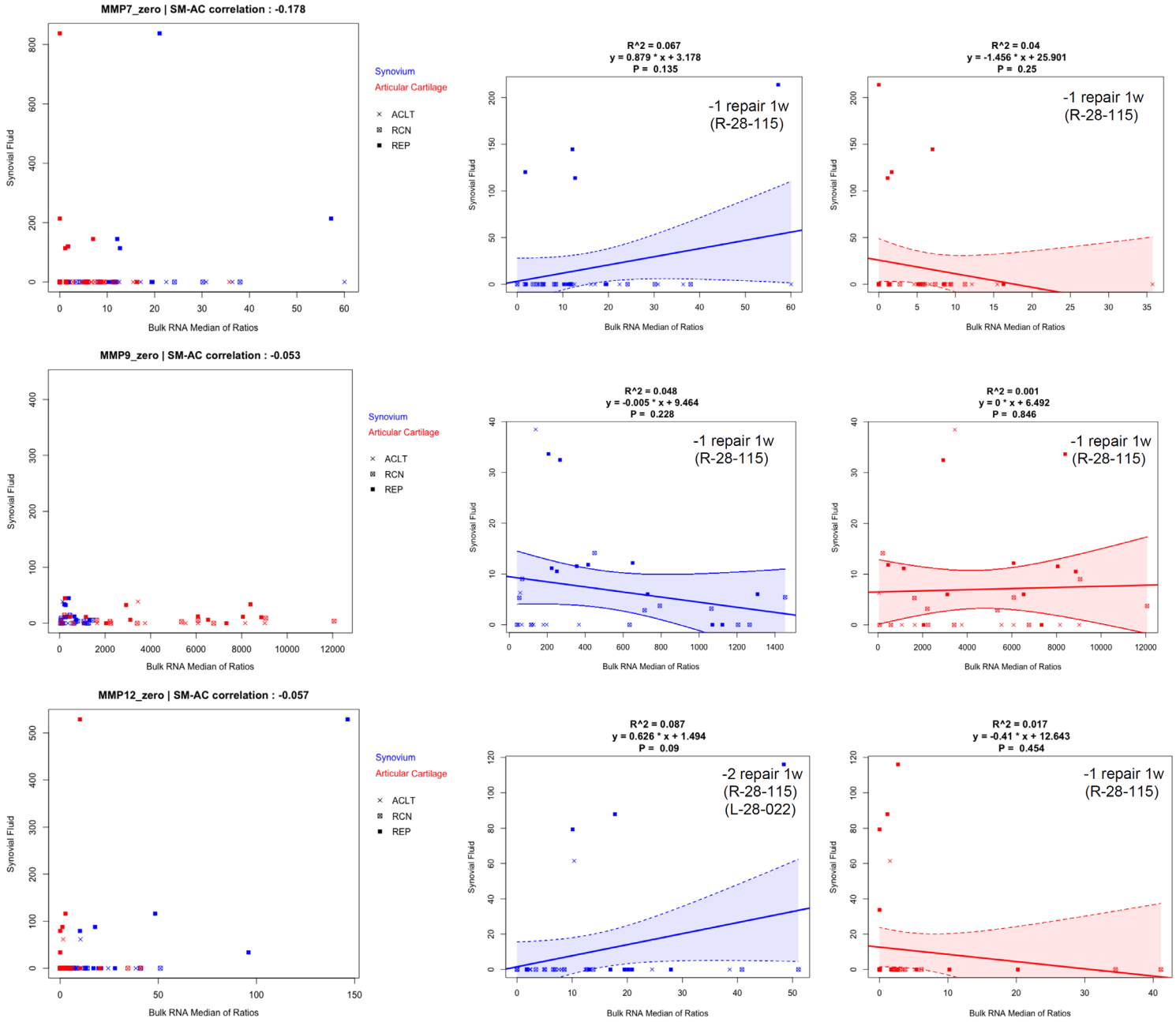








Gene and protein expression in the post-surgical joint



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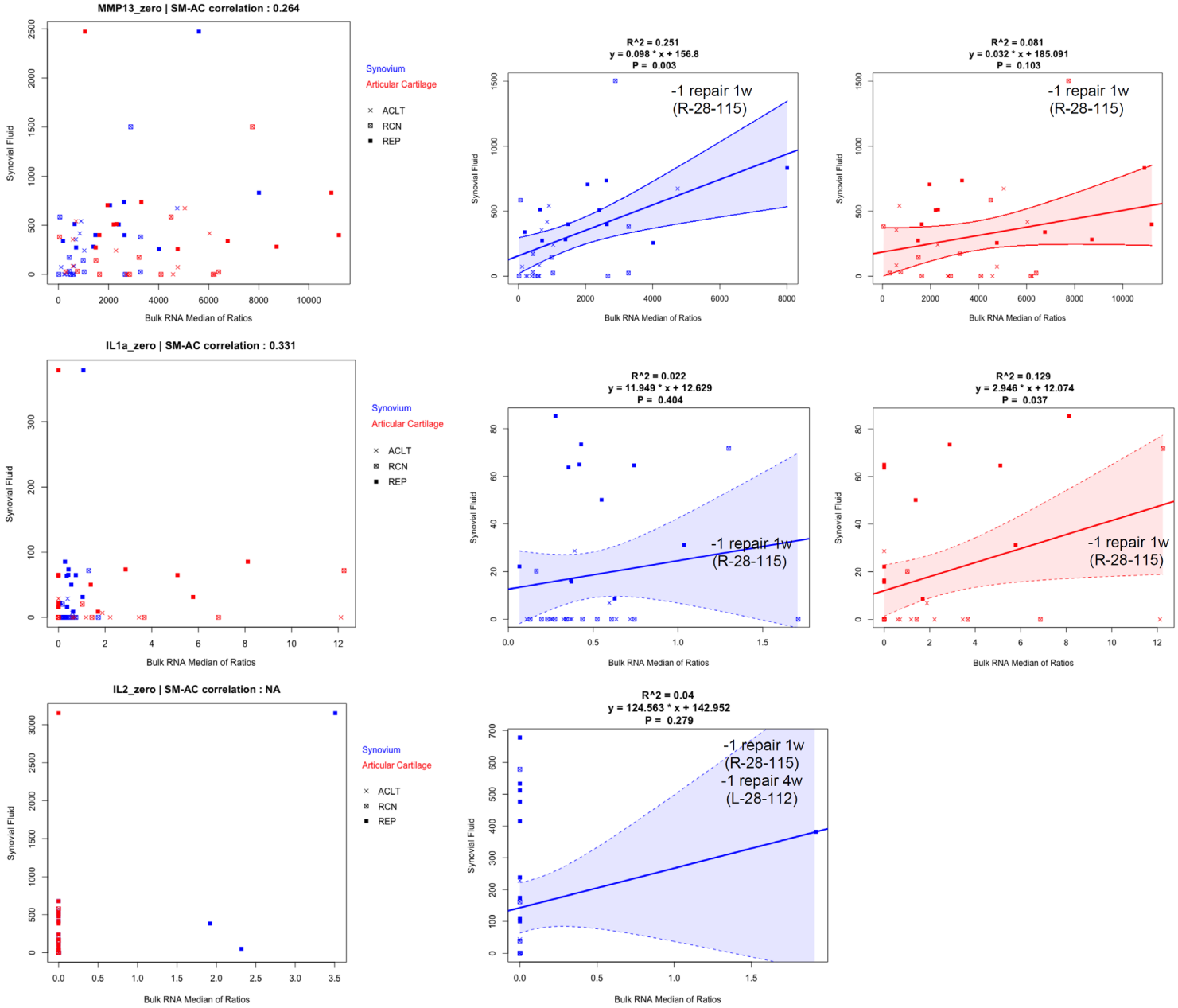

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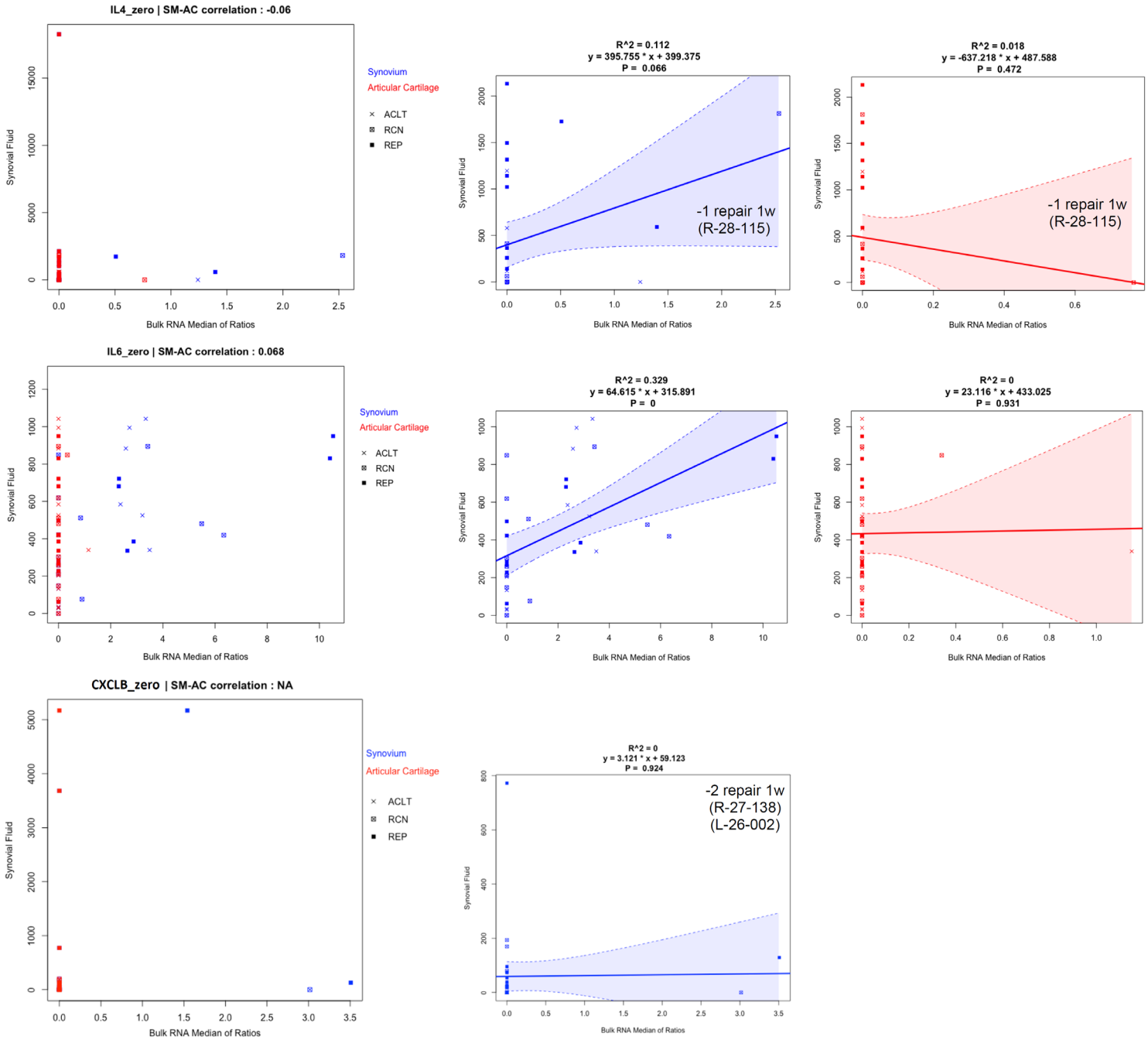

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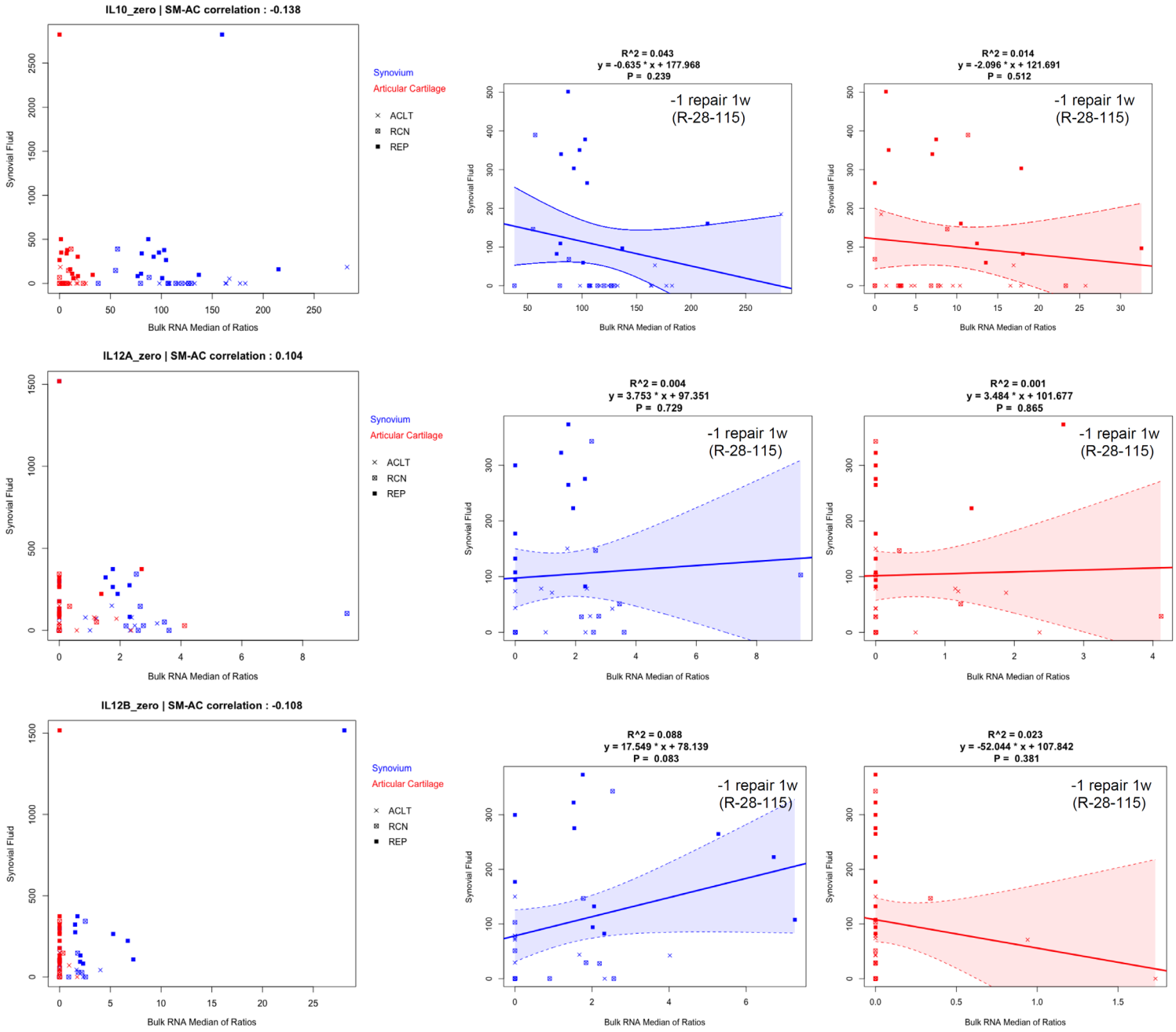

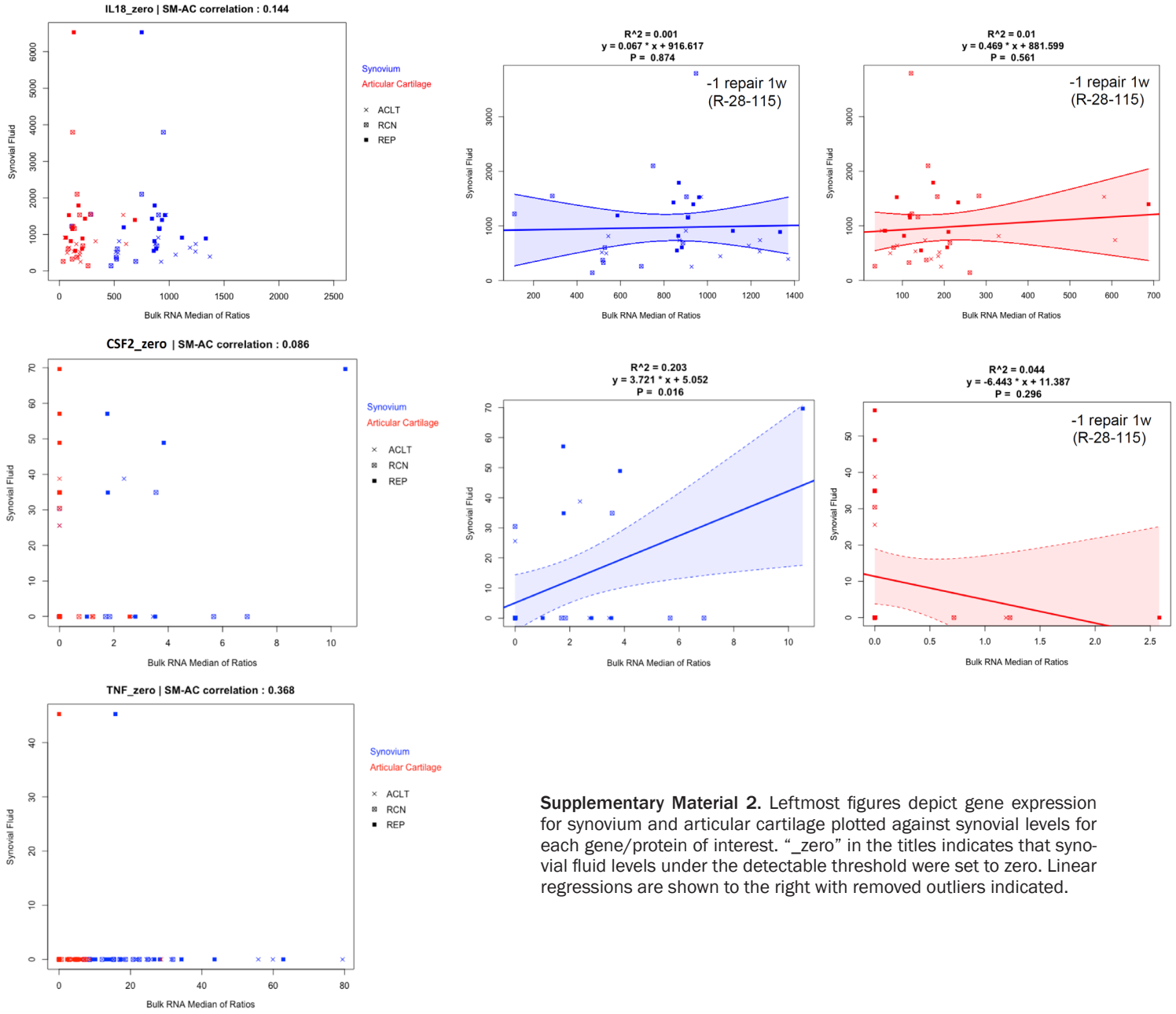

Supplementary Material 2. Leftmost figures depict gene expression for synovium and articular cartilage plotted against synovial levels for each gene/protein of interest. "_zero" in the titles indicates that synovial fluid levels under the detectable threshold were set to zero. Linear regressions are shown to the right with removed outliers indicated.

Table S1. Information is broken up by cytokines and MMPs of interest

A					
Protein	Age (mo)	Weight (kg)	Sex (F/M)	1 Week	4 Week
MMP1 (N=36)			18/18	N=18	N=18
mean	17.33	53.97			
stdev	1.23	3.61			
max/min	20.27/15.27	60/47			
MMP2 (N=31)			15/16	N=16	N=15
mean	17.22	54.32			
stdev	1.18	3.62			
max/min	19.37/15.27	60/47			
MMP3 (N=35)			18/17	N=18	N=17
mean	17.38	53.86			
stdev	1.2	3.6			
max/min	20.27/15.3	60/47			
MMP7 (N=36)			18/18	N=18	N=18
mean	17.33	53.97			
stdev	1.23	3.61			
max/min	20.27/15.27	60/47			
MMP9 (N=33)			18/15	N=18	N=15
mean	17.35	53.79			
stdev	1.23	3.7			
max/min	20.27/15.3	60/47			
MMP12 (N=36)			18/18	N=18	N=18
mean	17.33	53.97			
stdev	1.23	3.61			
max/min	20.27/15.3	60/47			
MMP13 (N=35)			18/17	N=18	N=17
mean	17.38	53.86			
stdev	1.2	3.6			
max/min	20.27/15.3	60/47			
B					
Protein	Age (mo)	Weight (kg)	Sex (F/M)	1 Week	4 Week
IL1a (N=35)			18/17	N=17	N=18
mean	17.36	54.09			
stdev	1.23	3.6			
max/min	20.27/15.27	60/47			
IL2 (N=33)			18/15	N=16	N=17
mean	17.48	53.79			
stdev	1.17	3.48			
max/min	20.27/15.3	60/47			
IL4 (N=32)			17/15	N=17	N=15
mean	17.36	53.81			
stdev	1.24	3.64			
max/min	20.27/15.3	60/47			
IL6 (N=36)			18/18	N=18	N=18
mean	17.33	53.97			
stdev	1.23	3.61			
max/min	20.27/15.27	60/47			

IL8 (N=33)			16/17	N=18	N=15
mean	17.41	53.88			
stdev	1.23	3.69			
max/min	20.27/15.3	60/47			
IL10 (N=35)			18/17	N=17	N=18
mean	17.31	53.89			
stdev	1.25	3.63			
max/min	20.27/15.27	60/47			
IL12B (N=36)			18/18	N=18	N=18
mean	17.33	53.97			
stdev	1.23	3.61			
max/min	20.27/15.27	60/47			
IL12A (N=36)			18/18	N=18	N=18
mean	17.33	53.97			
stdev	1.23	3.61			
max/min	20.27/15.27	60/47			
IL18 (N=36)			18/18	N=18	N=18
mean	17.33	53.97			
stdev	1.23	3.61			
max/min	20.27/15.27	60/47			
GM-CSF (N=28)			16/12	N=16	N=12
mean	17.48	53.75			
stdev	1.22	3.46			
max/min	20.27/15.27	60/47			
TNFα (N=35)			18/17	N=17	N=18
mean	17.31	53.8			
stdev	1.25	3.51			
max/min	20.27/15.27	60/47			

For protein groups in which all 36 samples had protein levels above the detectable limit, the same summary statistics are noted. (A) contains demographic data for animals whose synovial fluid samples had detectable levels of certain MMPs. (B) contains demographic data for animals whose synovial fluid samples had detectable levels of certain cytokines. A protein with less than 36 samples means that the Luminex assay deemed some sample(s) to have undetectable levels in the synovial fluid.

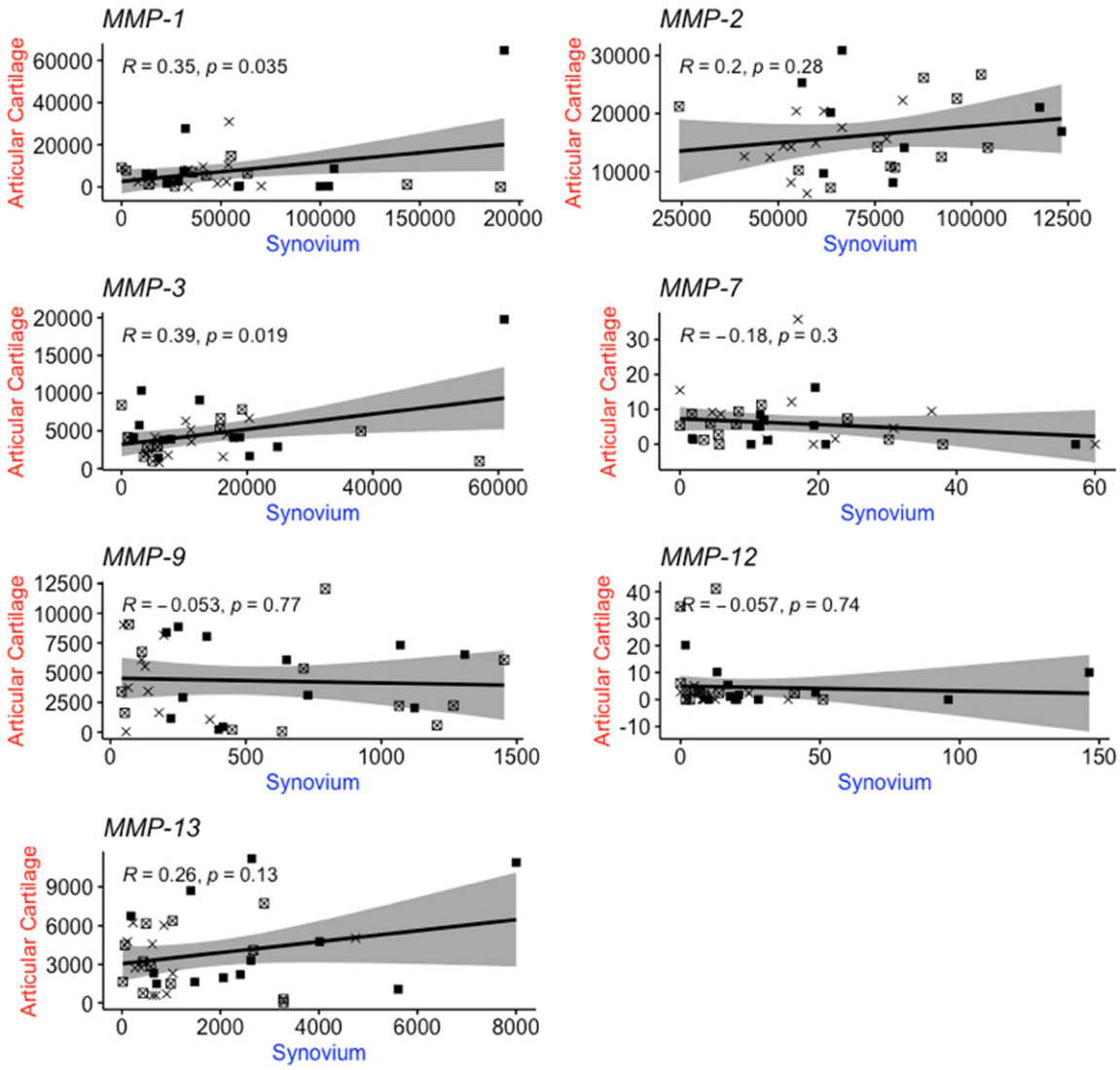

Figure S1. Of the seven MMPs evaluated, two featured significant correlations between gene expression in the articular cartilage and gene expression in the synovium. These MMPs were MMP-1 and MMP-3.

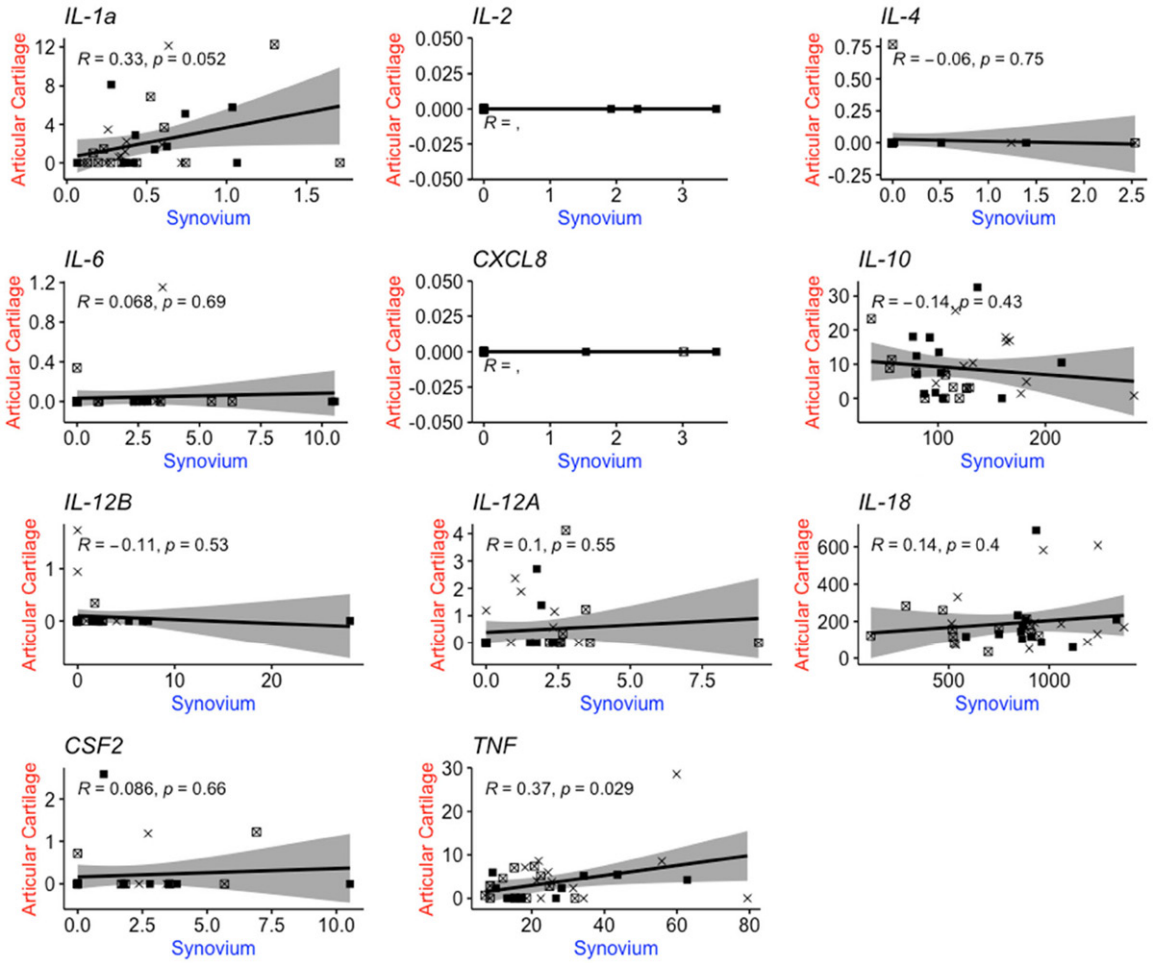

Figure S2. Of the eleven cytokines evaluated, two featured significant correlations between gene expression in the articular cartilage and gene expression in the synovium. These MMPs were IL-1a and TNF.

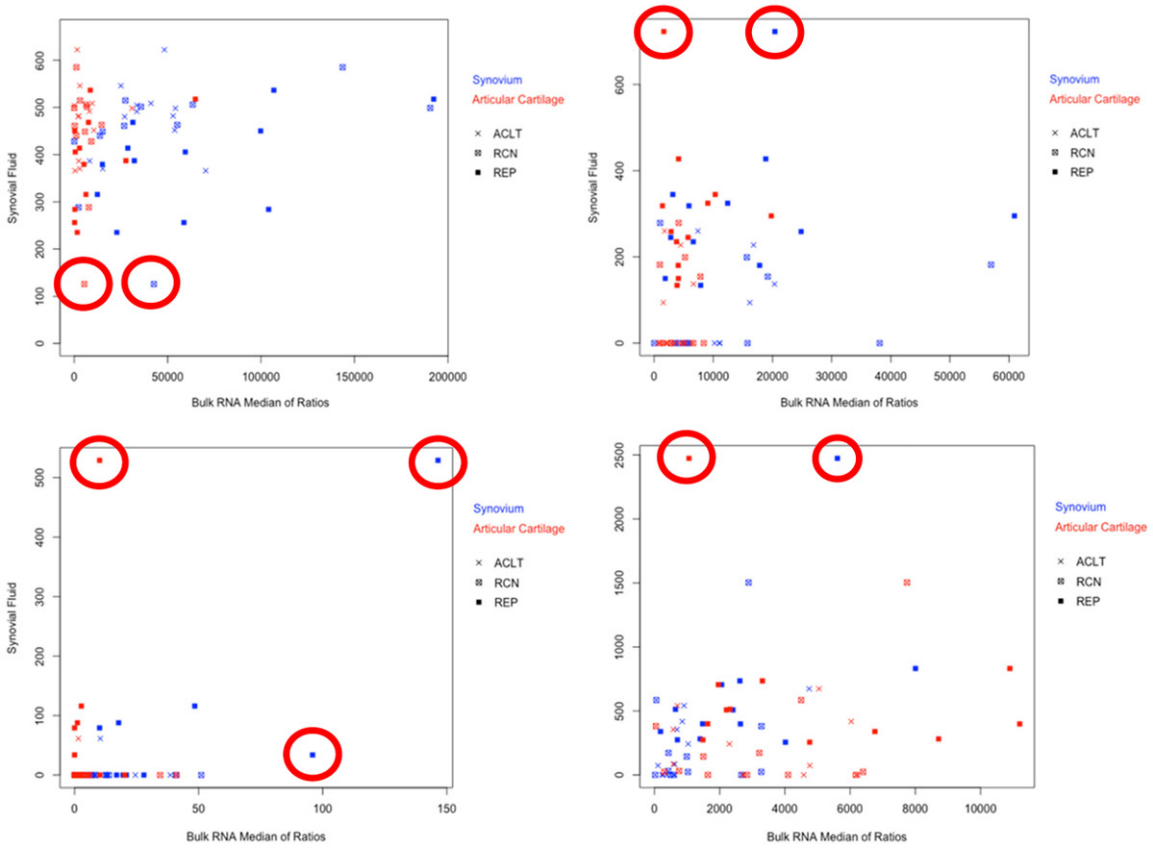

Figure S3. Of the seven MMPs evaluated, four featured significant regression relationships between synovial fluid protein levels and gene expression levels of at least one tissue. Gene expression in both tissues and synovial fluid proteins levels for those four MMPs are shown in this figure. Studentized residuals were calculated for each sample included in the bivariate regressions. Samples with abs (studentized residual) >3.0 are circled in red on this plot. These outlier samples were excluded from regression models.

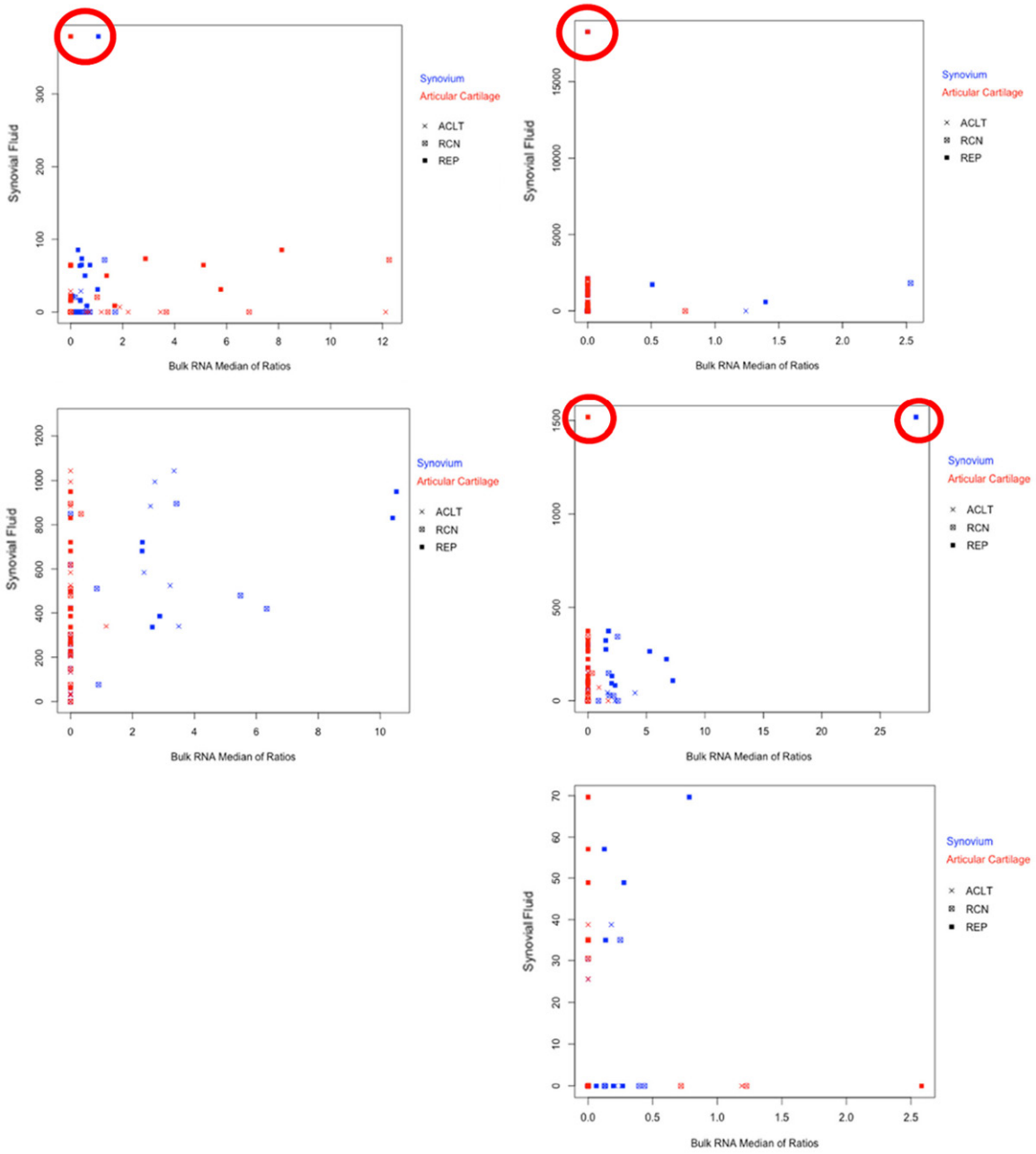

Figure S4. Of the eleven cytokines evaluated, five featured significant relationships between synovial fluid protein levels and gene expression levels of at least one tissue. Gene expression in both tissues and synovial fluid proteins levels for those five cytokines are shown in this figure. Gene expression in both tissues and synovial fluid proteins levels for those four MMPs are shown in this figure. Studentized residuals were calculated for each sample included in the bivariate regressions. Samples with abs (studentized residual) >3.0 are circled in red on this plot. These outlier samples were excluded from regression models.