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# Complement system dysregulation in synovial fluid from patients with persistent inflammation following anterior cruciate ligament reconstruction surgery

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## Abstract

**Introduction:** Patients with anterior cruciate ligament injury are at high risk of posttraumatic osteoarthritis and their response to reconstructive surgery and rehabilitation vary. Proteins identified in the orchestration of the acute inflammatory response may be predictive of patient outcomes.

**Objective:** An unbiased, bottom-up proteomics approach was used to discover novel targets for therapeutics in relation to dysregulation in the orchestration of inflammatory pathways implicated in persistent joint inflammation subsequent to joint trauma.

**Methods:** Synovial fluid was aspirated from patients at 1 week and 4 weeks after anterior cruciate ligament reconstruction (ACLR) and interleukin 6 (IL-6) concentrations were quantified by enzyme-linked immunosorbent assay. Patients were segregated into IL-$6^{low}$ and IL-$6^{high}$ groups based on IL-6 concentrations in synovial fluid at 4-weeks postoperation and proteins in synovial fluid were analyzed using qualitative, bottom-up proteomics. Abundance ratios were calculated for IL-$6^{high}$ and IL-$6^{low}$ groups as 4 weeks postoperation:1 week postoperation.



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Authorship contributions

L.E.K., L.A.F., C.L., E.R.H. and C.A.J. designed the study, analyzed the data, and drafted the manuscript. S.Z. and Q.F. contributed to study design, data interpretation and reviewed the manuscript. All authors approved the final version of the article.

Declaration of competing interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

Ethics approval

The University of Kentucky medical institutional review board approved this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi: 10.1016/j.jcjp.2023.100114.

**Results:** A total of 291 proteins were detected in synovial fluid, 34 of which were significantly ($P < .05$) differentially regulated between groups. Proteins associated with the classical and alternative complement cascade pathways were increased in the IL-$6^{high}$ compared to IL-$6^{low}$ group. Insulin-like growth factor-binding protein 6 (IGFBP-6) was increased by nearly 60-fold in the IL-$6^{low}$ group.

**Conclusions:** Patients segregated by IL-6 concentration in synovial fluid at 4 weeks post-ACLR demonstrated differential regulation of multiple pathways, providing opportunities to investigate novel targets, such as IGFBP-6, and to take advantage of therapeutics already approved for clinical use in other diseases that target inflammatory pathways, including the complement system.

### Keywords

Anterior cruciate ligament reconstruction; Complement; Inflammation; Interleukin 6; Posttraumatic osteoarthritis; Proteomic analysis

## Introduction

Posttraumatic osteoarthritis (PTOA) is the consequence of mechanical instability or joint trauma. Patients with anterior cruciate ligament (ACL) injury are at high risk of developing PTOA, with 50% to 60% of patients having radiographic changes consistent with osteoarthritis (OA) as early as 5 years, and clinical symptoms of OA within 10 years following injury. [1,2] Response to ACL reconstruction, rehabilitation, and treatment with anti-inflammatories has been met with varied results in the prevention of PTOA. [3,4] Traditional indices such as the extent of joint damage or patient pain have not been consistent predictors of success for the patient. There has been an increasing focus on personalized medicine approaches to treatment, but biomarkers need to first be identified.

Insights into why some patients recover while others with the same apparent magnitude of injury do not come from studies in polytrauma patients. Patients were identified as diverging into 2 subgroups during recovery, which have been variably termed susceptible/nonresponders and resistant/responders. These 2 groups could be identified based on their systemic inflammatory response in the acute stages following trauma, and define those who recover uneventfully (responders), and those who suffer from persistent inflammation (nonresponders). [5,6] These outcomes have been tied to temporal coordination of inflammatory responses, and patients who show early establishment and resolution of inflammation tend to have better outcomes. [7,8]

Similarly, there is increasing focus on the inflammatory response within the joint following injury and how the orchestration of this response is related to, and predictive of, patient outcomes. [9,10] Increased interleukin 6 (IL-6) within the joint has been detected in the acute and chronic phases of PTOA after ACL injury or patients with partial meniscectomy and is associated with progression of OA. [11,12] IL-6 signaling activates JAK proteins leading to downstream activation of STAT family proteins, YAP-NOTCH pathways, PI3K-Akt signaling, MAPK cascade, and activation of NF-kB. [13,14] Subsequently, IL-6 signaling leads to an inflammatory cascade, [15] as well as to further release of IL-6 in a positive feedback loop. [16] Continued elevation of IL-6 has also been indicated as a biomarker

of chronic inflammation in multiple other diseases including rheumatoid arthritis [17] and several types of cancer. [18,19] IL-6 could therefore be considered as a biomarker to identify ACL injury patients with persistent joint inflammation/nonresponders and those responder patients whose joint inflammation resolves after ACLR.

The purpose of this study was to identify proteins that are dysregulated early in the inflammatory process that may be targets for therapy in the prevention of PTOA. We hypothesized that these dysregulated pathways would be associated with expression of IL-6 and could be identified using an unbiased, bottom-up proteomics approach.

## Materials and methods

### Patients

Twenty-four patients with primary ACL injury consented to enrollment in an institutional review board-approved randomized clinical trial assessing the use of hyaluronate injection vs placebo 1-week postreconstruction (clinicaltrials.gov: NCT03429140). Patients were enrolled within the first 10 days following ACL injury. Group allotment was determined with a computerized randomization program, with group assignment being blinded in sealed envelopes until the patient arrived at their 1-week postoperative appointment. To be included in the original study, patients had to have an isolated ACL tear with no concurrent posterior cruciate ligament injury and could not have a grade 3 medial or lateral collateral ligament injury. Patients were between the ages of 14 and 32 and were skeletally mature with closed knee growth plates verified via radiograph. They had to have no history of previous surgery on the ipsilateral or contralateral knee and their ACL injury had to occur during sports activity. Exclusion criteria included the ACL injury occurring more than 10 days prior to enrollment, previous ipsilateral or contralateral knee surgery, intra-articular cortisone injection into either knee within 3 months of injury or a history of any inflammatory disease. The current study is a secondary analysis involving a subset of 16 patients that had successful joint aspirations at both the 1-week and 4-week postoperative time points.

### Study design

The current study is a secondary analysis of the previously mentioned randomized trial. One week postoperatively, patients received a knee aspiration, were randomized, and received an injection of either hyaluronate (Gel-One, Zimmer Biomet) or saline. Subsequent knee aspiration was then performed 4 weeks postreconstruction. Synovial fluid was centrifuged, aliquoted, and stored at −80 °C for further analysis. As was previously reported, there were no postoperative differences in synovial fluid IL-6 concentrations between the hyaluronate and saline groups, [11] and as such, data from all patients were pooled across groups for the current analyses.

### Biomarker assay

Synovial fluid IL-6 was assessed using a commercially available enzyme-linked immunosorbent assay (ELISA, Meso Scale Discovery). [11] The assay was run in duplicate, and any samples outside the limits of detection or quantifications were rerun and intra-assay coefficients of variance were less than 9.5 for all plates. The IL-6 ELISA was completed

per manufacturer guidelines. Based on IL-6 concentrations in synovial fluid at 4-weeks postreconstruction, patients were placed into either an IL-6 low (IL-$6^{low}$) group or IL-6 high (IL-$6^{high}$) group. A threshold value of synovial IL-6 was 316 pg/mL was used to define persistent inflammation within the joint, with 6 patients being placed into the IL-$6^{low}$ group and 10 patients placed in the IL-$6^{high}$ group.

### Bottom-up proteomics

The aim of this secondary analysis was to use an unbiased approach to identify proteins and pathways involved in persistent inflammation as potential targets for therapeutic intervention. Therefore, the proteomic study was designed to identify proteins that were differentially regulated between IL-$6^{low}$ and IL-$6^{high}$ groups. Absolute protein quantification was not performed, and abundance ratios were used to compare groups.

### In-gel trypsin digestion of sodium dodecyl sulfate gel bands

Fifty micrograms of proteins for each sample were loaded and separated on 10% BisTris sodium dodecyl sulfate gel under 2-(N-morpholino)ethanesulfonic acid buffer. A gel band covering 15 to 45 kD for each sample was excised, cut into ~1 mm cubes and subjected to in-gel digestion followed by extraction of the tryptic peptide as reported previously. [20] The excised gel pieces were washed consecutively in 200 μL distilled water, 100 mM ammonium bicarbonate (Ambic)/acetonitrile (1:1) and acetonitrile. The gel pieces were reduced with 70 μL of 10 mM DTT in 100 mM Ambic for 1 hour at 56°C and alkylated with 100 μL of 55 mM Iodoacetamide in 100 mM Ambic at room temperature in the dark for 60 minutes. After wash steps as described above, the gel slices were dried and rehydrated with 50 μL trypsin in 50 mM Ambic, 10% acetonitrile (ACN) (20 ng/μL) at 37°C for 16 hours. The digested peptides were extracted twice with 70 μl of 50% acetonitrile, 5% formic acid and once with 70 μl of 90% acetonitrile, 5% FA. Extracts from each sample were combined, filtered by a 0.22-μm spinning unit, and lyophilized.

### Protein identification by nano liquid chromatography-mass spectrophotometry (LC-MS/MS) analysis

The in-gel tryptic digests were reconstituted in 25 μL of 0.5% FA containing 125 fmol tryptic digest of yeast enolase for nano LC-ESI-MS/MS analysis, which was carried out using an Orbitrap Fusion Tribrid (Thermo-Fisher Scientific) mass spectrometer equipped with a nanospray Flex Ion Source, and coupled with a Dionex UltiMate3000RSLCnano system (Thermo-Fisher Scientific). [21,22] The gel extracted peptide samples (10 μL) were injected onto a PepMap C-18 RP nano trapping column (5 μm, 100 μm i.d × 20 mm) at 20 μL/min flow rate for rapid sample loading and then separated on a PepMap C-18 RP nano column (2 μm, 75 μm × 25 cm) at 35°C. The tryptic peptides were eluted in a 120-minute gradient of 5% to 35% ACN in 0.1% formic acid at 300 nL/min, followed by a 7-minute ramping to 90% ACN-0.1% FA and an 8-minute hold at 90% ACN-0.1% FA. The column was re-equilibrated with 0.1% FA for 25 minutes prior to the next run. The Orbitrap Fusion is operated in positive ion mode with spray voltage set at 1.6 kV and source temperature at 275°C. External calibration for Fourier transform, ion trap and quadrupole mass analyzers was performed. In data-dependent acquisition (DDA) analysis, the instrument was operated using Fourier transform mass analyzer in MS scan to select precursor ions followed by 3

second “Top Speed” data-dependent collision-induced dissociation ion trap MS/MS scans at 1.6 m/z quadrupole isolation for precursor peptides with multiple charged ions above a threshold ion count of 10,000 and normalized collision energy of 30%. MS survey scans at a resolving power of 120,000 (fwhm at m/z 200), for the mass range of m/z 375 to 1575. Dynamic exclusion parameters were set at 40 seconds of exclusion duration with ± 10 ppm exclusion mass width. All data were acquired under Xcalibur 4.3 operation software (Thermo-Fisher Scientific).

## Data analysis

The DDA raw files for collision-induced dissociation MS/MS were subjected to database searches using Proteome Discoverer (PD) 2.4 software (Thermo-Fisher Scientific) with the Sequest HT algorithm processing workflow for precursor-based quantification. The PD 2.4 processing workflow containing an additional node of Minora Feature Detector for precursor ion-based quantification was used for protein identification and protein relatively quantitation analysis between samples. The database search was conducted against a *Homo sapiens* database containing 81,785 sequences downloaded from the National Center for Biotechnology Information. Two-missed trypsin cleavage sites were allowed. The peptide precursor tolerance was set to 10 ppm and fragment ion tolerance was set to 0.6 Da. Variable modification of methionine oxidation, deamidation of asparagines/glutamine, and fixed modification of cysteine carbamidomethylation were set for the database search. Identified peptides were further filtered for maximum 1% false discovery rate using the Percolator algorithm in PD 2.4 along with additional peptide confidence set to high and peptide mass accuracy ≤ 5 ppm. The final protein identifiers contained protein groups that were filtered with at least 2 peptides per protein. Relative quantitation of identified proteins between the paired samples for each of the 16 individuals was determined by the Label Free Quantitation workflow in PD 2.4. The precursor abundance intensity for each peptide identified by MS/MS in each sample was automatically determined and their unique plus razor peptides for each protein in each sample were summed, normalized against yeast enolase protein, and used for calculating the protein abundance by PD 2.4 software. Protein ratios were calculated based on pairwise ratio for the 2 data points of samples. Results were further analyzed using Protein Analysis Through Evolutionary Relationships (PANTHER), [23] Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) software, [24] and DAVID (Database for Annotation, Visualization and Integrated Discovery) [25] functional annotation clustering.

## Protein selection

Albumin and redundant proteins were not included in further analysis. Proteins of interest were identified as being significantly different between the 2 groups using a t-test with $P < .05$. Protein selection was further refined to include those having an absolute difference of abundance ratio between IL-$6^{low}$ and IL-$6^{high}$ groups of greater than 0.8, and proteins with an absolute difference of abundance ratio of less than 0.8 but were differentially up- or down-regulated between IL-$6^{low}$ and IL-$6^{high}$ groups.

## Results

### LC-MS/MS analysis of synovial fluid post-ACLR

To identify novel targets in synovial fluid that may contribute to persistence of articular inflammation following ACL injury, LC-MS/MS was used in an unbiased approach. Pilot studies revealed heavy contamination of albumin at >50 kDa and potential albumin degradation product at <15 kDa, especially in patients within the IL-$6^{high}$ group. Therefore, only proteins within the 15 to 45 kDa range were analyzed.

A total of 291 proteins were confidently identified in synovial fluid samples. The complete list can be found in Table S1. Pathway analysis by PANTHER revealed that the proteins detected were associated with 19 protein classes (Fig. 1). The majority (59%) fell under 3 classifications: metabolite interconversion enzymes (20%), protein modifying enzymes (21%), protein-binding activity modulators (18%). Metabolite interconversion enzymes included proteins related to mitochondrial function and glycolysis. Protein modifying enzymes included proteases and proteins related to the complement system. Protein-binding activity modulators also included proteins of the complement system, as well as protease inhibitors. Extracellular matrix proteins (9%) were in fourth highest abundance.

### Proteins in synovial fluid differentially regulated in persistent inflammation

From the full list of 291 proteins that were identified, 34 were identified as proteins of interest (Fig. 2). A repeat PANTHER analysis on the proteins of interest reduced the number of protein classes to 11 from 19 (Fig. 3). The predominant classes remained protein-binding activity enzymes (26.9%) and protein-modifying enzymes (16.4%).

Of the 34 proteins of interest, 27 (80%) were differentially up-regulated in the IL-$6^{high}$ group (Table 1) and 20% were downregulated (Table 2). Analysis of predicted protein-protein interactions by STRING showed that these proteins are highly related, with a total of 44 edges, which are protein-protein associations (Fig. 4; protein-protein interaction enrichment $P < 1.0e^{-16}$). Proteins from all activation pathways of the complement system were detected and were represented in 7/8 Kyoto Encyclopedia of Genes and Genetics database pathways identified (Table 3). The complement cascade pathway accounted for 25.9% of the proteins upregulated in the IL-$6^{high}$ group with a Benjamini-corrected $P$ value of $1.1e^{-7}$.

### Proteins in synovial fluid upregulated in inflammation resolution

Only 7 of the 34 proteins of interest were upregulated in the IL-$6^{low}$ group (Table 4), with no proteins being significantly downregulated in this group at 4 weeks compared to the IL-$6^{high}$ group. Insulin-like growth factor binding protein 6 (IGFBP-6), which primarily binds to and blocks the actions of insulin-like growth factor-II (IGF-II), but also has IGF-independent actions, was the most highly upregulated protein in the IL-$6^{low}$ group. The abundance of IGFBP-6 was increased by nearly 60-fold in IL-$6^{low}$ patients, and was decreased in IL-$6^{high}$ patients, suggesting it may be involved in resolution of inflammation. Types V and VI collagen were upregulated and were highly related in the STRING analysis (Fig. 5; protein-protein interaction enrichment $P < 5e^{-6}$) and were the only proteins present in all 4

Kyoto Encyclopedia of Genes and Genetics pathways identified by DAVID analysis (Table 5; Benjamini-corrected $P < .05$).

## Discussion

The results of this study reveal that multiple protein classes are differentially regulated between patients with high vs low concentrations of IL-6 in the synovial fluid at 4 weeks post-ACLR surgery. Those with high synovial fluid IL-6 are presumed to have persistent inflammation, and patients with low IL-6 are presumed to demonstrate early resolution of inflammation. Patients in the IL-$6^{high}$ group had an increase in abundance of proteins associated with the complement system, which offer therapeutic targets early in PTOA in the prevention of persistent inflammation following injury.

Two pathways of the complement system, classical and alternative, have been recognized as components of the inflammatory response in PTOA and contribute to the release of leukocyte chemoattractants. [26] While the complement system also contributes to the clearance of dead and dying cells, aiding in repair of damaged tissue, its persistence within the joint contributes to hypertrophy of chondrocytes and their transdifferentiation into osteoblasts, contributing to joint destruction. [27] Complement components also play a role in mediating catabolic enzymes, including MMPs and ADAMTSs, and inflammatory cytokines such as CCL2, CCL5, and CSF1.[27] This highlights the complement system as a pathway of inflammatory dysregulation, as multiple complement inhibitors, including eculizumab, and C1 esterase inhibitors, have already been approved by the Food and Drug Administration for diseases such as paroxysmal nocturnal hemoglobinuria, neuromyelitis optica, and angioedema. [28]

Furthermore, collectin 11, a soluble C-type lectin, binds to L-fucose in the surface of cells in the presence of cell stress to initiate activation of complement, leading to tissue injury. [29] In contrast, collectin-11 also plays a role in the suppression of antigen-presenting cell activation and function, and reduced clinical arthritis scores, disease incidence, and histopathology scores in a collagen-induced mouse. Although collectin-11 is clearly linked to complement cascade activation, further exploration of its role in the induction and progression of PTOA is needed before exploring it as a therapeutic target.

Members of the serpin superfamily, which includes heparin cofactor 2 (serpin D 1) and protein Z-dependent protease (serpin A 10) are highly associated with the complement system in other disease systems, including cancer. Heparin cofactor 2 and complement C4B, which are increased in the synovial fluid IL-$6^{high}$ nonresponders within this study, are increased in the serum of patients with nasopharyngeal carcinoma who do not respond to radiotherapy. [30] This may indicate heparin cofactor 2 and C4B as biomarkers in synovial fluid of patients who may be predisposed to persistent inflammation after ACLR. Protein Z-dependent protease and complement C3 were identified as factors associated with colorectal cancer metastasis to the liver, again indicating members of the serpin superfamily as potential inflammatory biomarkers in PTOA development.

The alternate approach to restoring balance within the joint, rather than blocking aberrant inflammatory responses, would be to increase anti-inflammatory responses. Within the IL-6$^{low}$ group, IGFBP-6 was increased by nearly 60-fold, whereas it was decreased by 0.28-fold in the IL-6$^{high}$ group. IGFBP-6 is an acute-phase protein [31] that binds to IGF-II over IFG-I, and inhibits its effector functions associated with differentiation, proliferation, and survival of a variety of cell types. [32] IGF-independent functions of IGFBP-6 include induction of migration of monocyte and T cells, [33,34] both of which have subtypes that participate in wound healing and immunoregulation. IGFBP-6 is also highly expressed in fibroblasts and plays a role in the maintenance of collective tissue. [35] These data warrant further investigation of the role of IGFBP-6 in tissue injury response within the joint as a potential therapeutic target in the mitigation of PTOA following ACLR.

There are several limitations to this study. First, this study involved samples from 16 patients and the small sample size could affect both the internal validity and generalizability of the study's findings. Future work with larger sample sizes and more broad inclusion and exclusion criteria are needed to validate the current results. Second, there is no validated synovial IL-6 concentration that has been found to be predictive of progressive cartilage degradation after ACLR. The 316 pg/mL threshold was based on previous literature, [11] but future work is necessary to determine if a true threshold value can be determined. While bottom-up proteomics provides an unbiased approach for the discovery of therapeutic targets in PTOA, contamination of synovial fluid with albumin, keratins and fibrinogens suppressed detection of lower abundance proteins, [36] including interleukins, such as IL-6, which are key to the orchestration of inflammation resolution. [5,37] Bottom-up proteomics also provides qualitative data rather than quantitative, and so comparisons between groups are limited. An alternative approach would be a targeted, multiplex ELISA with computational modeling and analysis. [6] Analysis of synovial fluid provides identification of proteins, but not their source. Pairing proteomic analysis with flow cytometry analysis or single cell RNA sequencing of cells present within the synovial fluid would provide a more comprehensive view on cellular targets for therapeutics in the mitigation of persistent inflammation leading to PTOA following traumatic joint injury.

## Conclusions

In summary, patients segregated based on IL-6 concentrations in synovial fluid 4 weeks post-ACLR demonstrated differential regulation of multiple inflammatory pathways. These findings highlight the need for well-powered, longitudinal studies to determine if patient-specific therapies that target the correct molecule at the right time may be able to alter PTOA progression prior to the inevitability of joint destruction.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

1. Lohmander LS, Östenberg A, Englund M, Roos H. High prevalence of knee osteoarthritis, pain, and functional limitations in female soccer players twelve years after anterior cruciate ligament injury. Arthritis Rheum. 2004;50(10):3145–3152. doi: 10.1002/art.20589. [PubMed: 15476248]
2. Buller LT, Best MJ, Baraga MG, Kaplan LD. Trends in anterior cruciate ligament reconstruction in the United States. Orthop J Sports Med. 2015;3(1):1–8. doi: 10.1177/2325967114563664.
3. Lattermann C, Jacobs CA, Proffitt Bunnell M, et al. A multicenter study of early anti-inflammatory treatment in patients with acute anterior cruciate ligament tear. Am J Sports Med. 2017;45(2):325–333. doi: 10.1177/0363546516666818. [PubMed: 28146402]
4. Wang LJ, Zeng N, Yan ZP, Li JT, Ni GX. Post-traumatic osteoarthritis following ACL injury. Arthritis Res Ther. 2020;22(1):57. doi:10.1186/s13075-020-02156-5. [PubMed: 32209130]
5. Namas RA, Almahmoud K, Mi Q, et al. Individual-specific principal component analysis of circulating inflammatory mediators predicts early organ dysfunction in trauma patients. J Crit Care. 2016;36:146–153. doi: 10.1016/j.jcrc.2016.07.002. [PubMed: 27546764]
6. McKinley TO, Gaski GE, Zamora R, et al. Early dynamic orchestration of immunologic mediators identifies multiply injured patients who are tolerant or sensitive to hemorrhage. J Trauma Acute Care Surg. 2021;90(3):441–450. doi: 10.1097/TA.0000000000002998. [PubMed: 33093290]
7. Almahmoud K, Abboud A, Namas RA, et al. Computational evidence for an early, amplified systemic inflammation program in polytrauma patients with severe extremity injuries. PLoS One. 2019;14(6):e0217577. doi: 10.1371/journal.pone.0217577. [PubMed: 31163056]
8. Lamparello AJ, Namas RA, Constantine G, et al. A conceptual time window-based model for the early stratification of trauma patients. J Intern Med. 2019;286(1):2–15. doi: 10.1111/joim.12874. [PubMed: 30623510]
9. King JD, Rowland G, Villasante Tezanos AG, et al. Joint fluid proteome after anterior cruciate ligament rupture reflects an acute posttraumatic inflammatory and chondrodegenerative state. Cartilage. 2020;11(3):329–337. doi: 10.1177/1947603518790009. [PubMed: 30033738]
10. Larsson S, Struglics A, Lohmander LS, Frobell R. Surgical reconstruction of ruptured anterior cruciate ligament prolongs trauma-induced increase of inflammatory cytokines in synovial fluid: an exploratory analysis in the KANON trial. Osteoarthritis Cartilage. 2017;25(9):1443–1451. doi:10.1016/j.joca.2017.05.009. [PubMed: 28522220]
11. Hunt ER, Jacobs CA, Conley CEW, Ireland ML, Johnson DL, Lattermann C. Anterior cruciate ligament reconstruction reinitiates an inflammatory and chondrodegenerative process in the knee joint. J Orthop Res. 2020;39(6):1–8. doi: 10.1002/jor.24783.
12. Larsson S, Englund M, Struglics A, Lohmander LS. Interleukin-6 and tumor necrosis factor alpha in synovial fluid are associated with progression of radiographic knee osteoarthritis in subjects with previous meniscectomy. Osteoarthritis Cartilage. 2015;23(11):1906–1914. doi:10.1016/j.joca.2015.05.035. [PubMed: 26521736]
13. Taniguchi K, Wu LW, Grivennikov SI, et al. A gp130–Src–YAP module links inflammation to epithelial regeneration. Nature. 2015;519(7541). doi:10.1038/nature14228.
14. Kang S, Narazaki M, Metwally H, Kishimoto T. Historical overview of the interleukin-6 family cytokine. J Exp Med. 2020;217(5):e20190347. doi: 10.1084/jem.20190347. [PubMed: 32267936]
15. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol. 2014;6(10):a016295. doi:10.1101/cshperspect.a016295. [PubMed: 25190079]

16. Lee J, Nakagiri T, Oto T, et al. IL-6 Amplifier, NF-κB–triggered positive feedback for IL-6 signaling, in grafts is involved in allogeneic rejection responses. J Immunol. 2012;189(4):1928–1936. doi: 10.4049/jimmunol.1103613. [PubMed: 22798669]

17. Hashizume M, Mihara M. The roles of interleukin-6 in the pathogenesis of rheumatoid arthritis. Arthritis. 2011;2011:1–8. doi:10.1155/2011/765624.

18. Grivennikov S, Karin E, Terzic J, et al. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell. 2009;15(2):103–113. doi: 10.1016/j.ccr.2009.01.001. [PubMed: 19185845]

19. Masjedi A, Hashemi V, Hojjat-Farsangi M, et al. The significant role of interleukin-6 and its signaling pathway in the immunopathogenesis and treatment of breast cancer. Biomed Pharmacother. 2018;108:1415–1424. doi: 10.1016/j.biopha.2018.09.177. [PubMed: 30372844]

20. Yang Y, Thannhauser TW, Li L, Zhang S. Development of an integrated approach for evaluation of 2-D gel image analysis: impact of multiple proteins in single spots on comparative proteomics in conventional 2-D gel/MALDI workflow. Electrophoresis. 2007;28(12):2080–2094. doi:10.1002/elps.200600524. [PubMed: 17486657]

21. Yang Y, Anderson E, Zhang S. Evaluation of six sample preparation procedures for qualitative and quantitative proteomics analysis of milk fat globule membrane. Electrophoresis. 2018;39(18):2332–2339. doi: 10.1002/elps.201800042. [PubMed: 29644703]

22. Thomas CJ, Cleland TP, Zhang S, Gundberg CM, Vashishth D. Identification and characterization of glycation adducts on osteocalcin. Anal Biochem. 2017;525:46–53. doi: 10.1016/j.ab.2017.02.011. [PubMed: 28237256]

23. Mi H, Muruganujan A, Thomas PD. PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. Nucleic Acids Res. 2012;41(D1):D377–D386. doi: 10.1093/nar/gks1118. [PubMed: 23193289]

24. Szklarczyk D, Franceschini A, Wyder S, et al. STRING v10: protein–protein interaction networks, integrated over the tree of life. Nucleic Acids Res. 2015;43(D1):D447–D452. doi: 10.1093/nar/gku1003. [PubMed: 25352553]

25. Dennis G, Sherman BT, Hosack DA, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. 2003;4:R60. doi: 10.1186/gb-2003-4-9-r6.

26. Jonh T, Stahel PF, Morgan SJ, Schulze-Tanzil G. Impact of the complement cascade on posttraumatic cartilage inflammation and degradation. Histol Histopathol. 2007;22(7–9):781–790. doi: 10.14670/HH-22.781. [PubMed: 17455152]

27. Riegger J, Brenner RE. Pathomechanisms of posttraumatic osteoarthritis: chondrocyte behavior and fate in a precarious environment. Int J Mol Sci. 2020;21(5):1560. doi: 10.3390/ijms21051560. [PubMed: 32106481]

28. Zipfel PF, Wiech T, Rudnick R, Afonso S, Person F, Skerka C. Complement inhibitors in clinical trials for glomerular diseases. Front Immunol. 2019;10:2166. doi: 10.3389/fimmu.2019.02166. [PubMed: 31611870]

29. Farrar CA, Tran D, Li K, et al. Collectin-11 detects stress-induced L-fucose pattern to trigger renal epithelial injury. J Clin Invest. 2016;126(5):1911–1925. doi: 10.1172/JCI83000. [PubMed: 27088797]

30. Zhang G, Zhang K, Li C, et al. Serum proteomics identify potential biomarkers for nasopharyngeal carcinoma sensitivity to radiotherapy. Biosci Rep. 2019;39(5):BSR20190027. doi: 10.1042/BSR20190027. [PubMed: 31040200]

31. Conese M, Pace L, Pignataro N, et al. Insulin-like growth factor binding protein 6 is secreted in extracellular vesicles upon hyperthermia and oxidative stress in dendritic cells but not in monocytes. Int J Mol Sci. 2020;21(12):1–10. doi: 10.3390/ijms21124428.

32. Bach LA. Recent insights into the actions of IGFBP-6. J Cell Commun Signal. 2015;9(2):189–200. doi:10.1007/s12079-015-0288-4. [PubMed: 25808083]

33. Alunno A, Bistoni O, Manetti M, et al. Insulin-like growth factor binding protein 6 in rheumatoid arthritis: a possible novel chemotactic factor? Front Immunol. 2017;8:554. doi: 10.3389/fimmu.2017.00554. [PubMed: 28572803]

34. Liso A, Capitanio N, Gerli R, Conese M. From fever to immunity: a new role for IGFBP-6? J Cell Mol Med. 2018;22(10):4588–4596. doi:10.1111/jcmm.13738. [PubMed: 30117676]

35. Liso A, Venuto S, Coda ARD, Giallongo C, Palumbo GA, Tibullo D. IGFBP-6: at the crossroads of immunity, tissue repair and fibrosis. Int J Mol Sci. 2022;23(8):4358. doi: 10.3390/ijms23084358. [PubMed: 35457175]

36. Pietrowska M, Wlosowicz A, Gawin M, Widlak P. MS-based proteomic analysis of serum and plasma: problem of high abundant components and lights and shadows of albumin removal. In: Capelo-Martínez JL, ed. Emerging Sample Treatments in Proteomics. Advances in Experimental Biology. Cham: Springer; 2019:57–76. doi: 10.1007/978-3-030-12298-0_3.

37. Maier B, Lefering R, Lehnert M, et al. Early versus late onset of multiple organ failure is associated with differing patterns of plasma cytokine biomarker expression and outcome after severe trauma. Shock. 2007;28(6):668–674. doi: 10.1097/shk.0b013e318123e64e. [PubMed: 18092384]

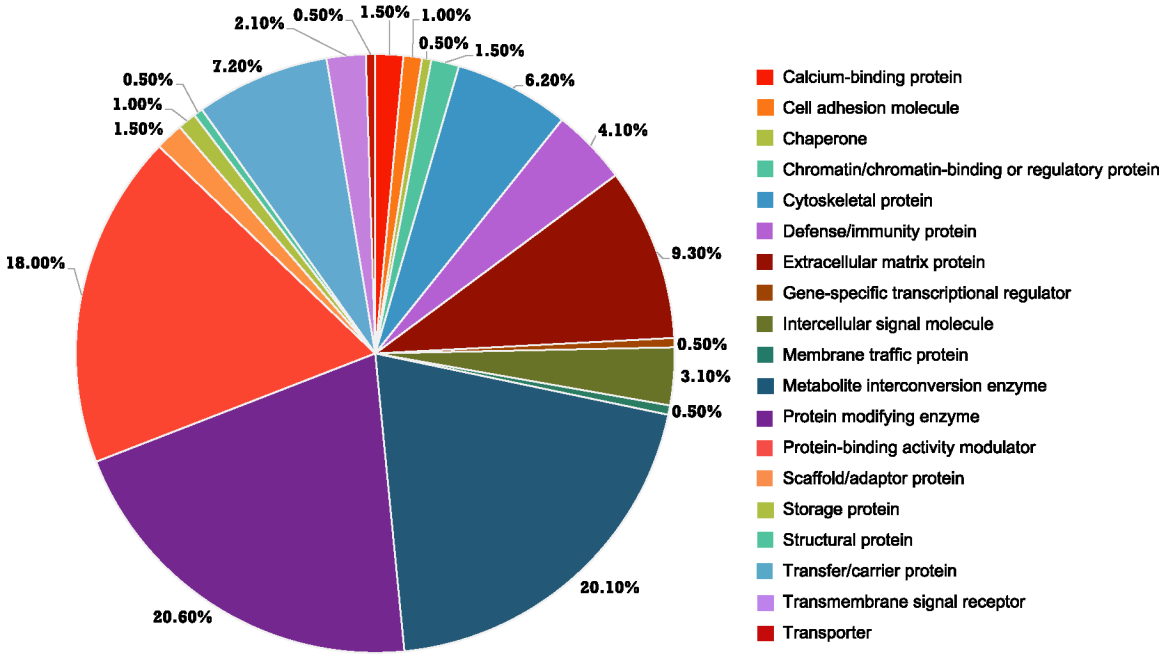


**Fig. 1.**

Protein Analysis Through Evolutionary Relationships analysis revealed 19 classes of proteins present in the synovial fluid following anterior cruciate ligament injury. Proteins of high abundance in synovial fluid are largely involved in protein modification through enzymatic or protein-binding functions.

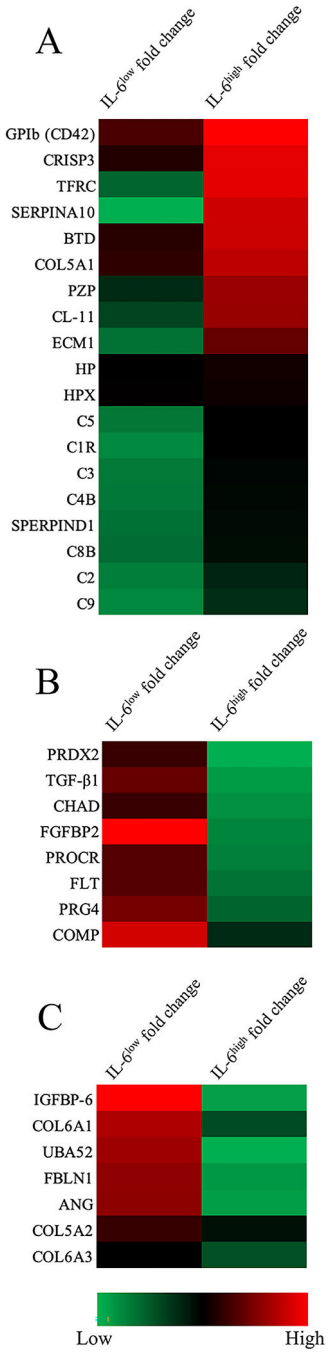


**Fig. 2.**

Heat map of protein abundance fold change of the 34 different abundance proteins of interest that are increased in abundance in the IL-6$^{high}$ 378 group (**A**), decreased in abundance in the IL-6$^{high}$ group (**B**), and increased in abundance in the IL-6$^{low}$ 379 group (**C**). IL-6, interleukin 6.

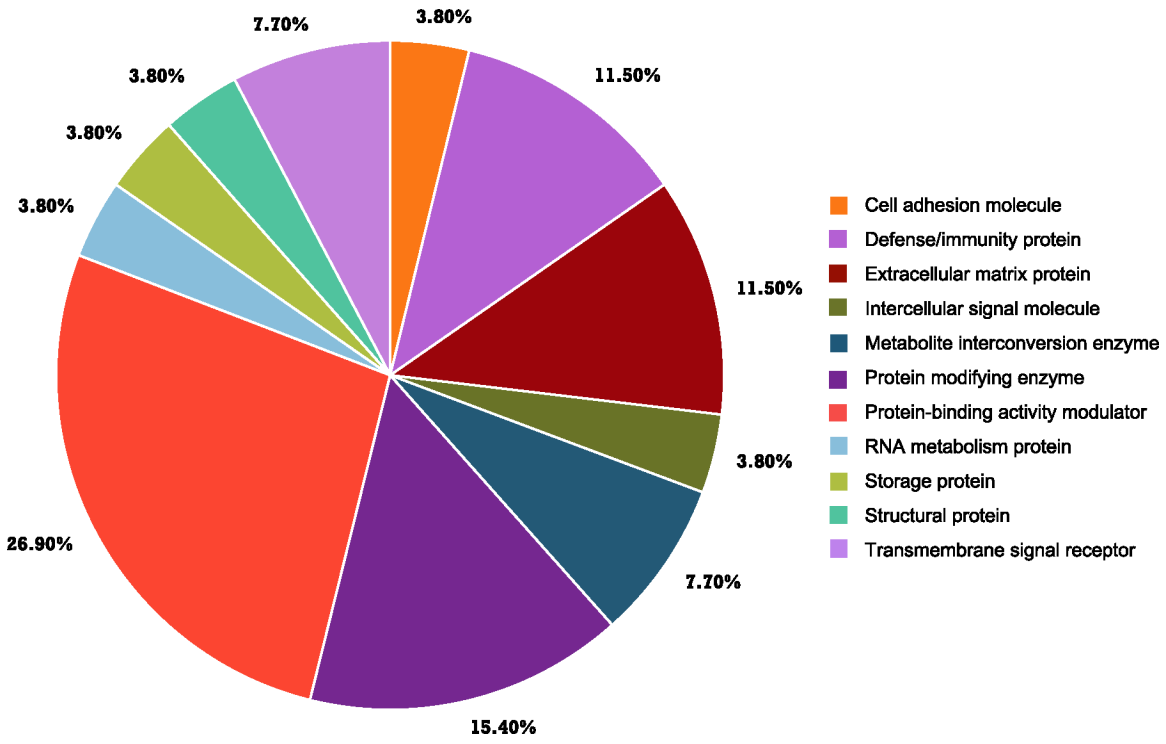


**Fig. 3.**

Protein Analysis Through Evolutionary Relationships analysis of proteins of interest reduces number protein classes to 11. The dominant protein classes following reduction remain as metabolite interconversion enzymes, protein modifying enzymes, and protein-binding activity modulators. The percent of proteins that are related to defense/immunity more than doubled following protein list refinement.

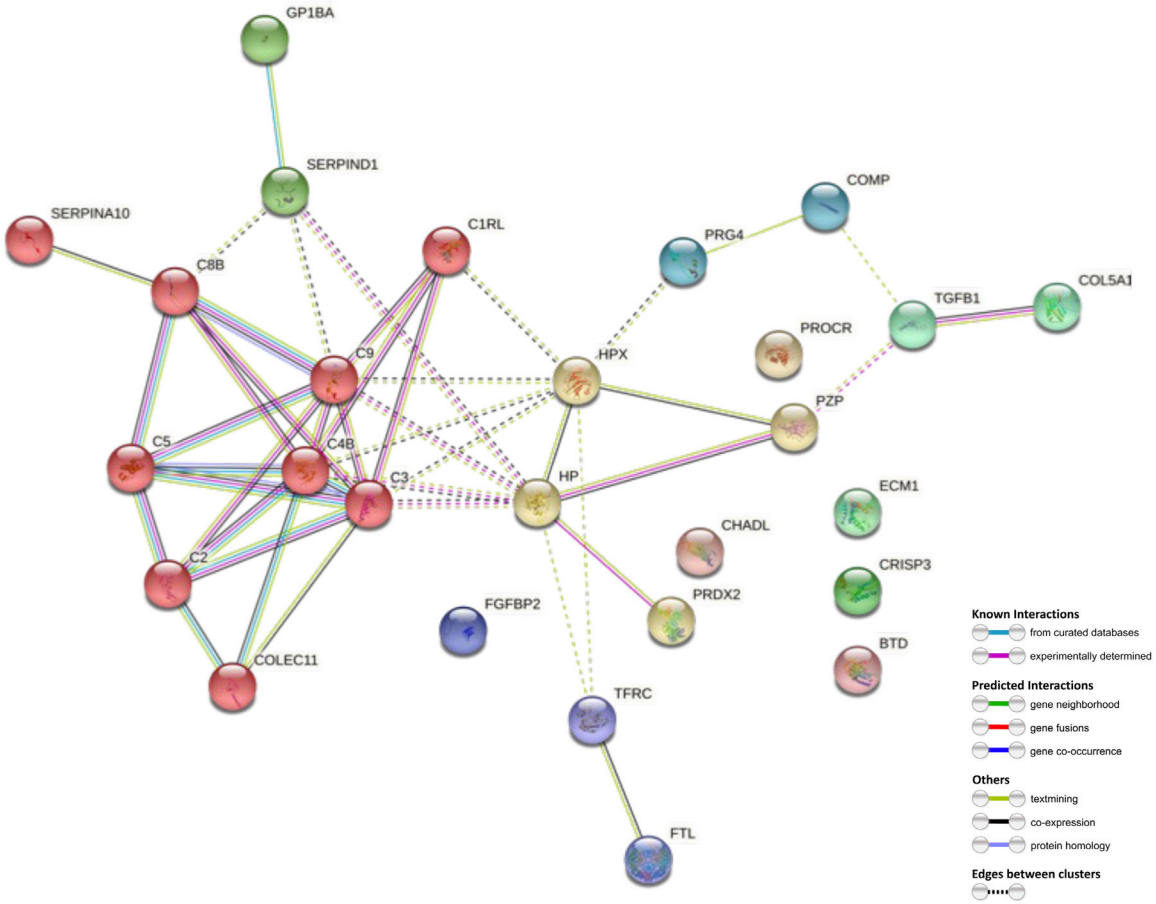


**Fig. 4.**

Analysis of protein-protein interactions by Search Tool for the Retrieval of Interacting Genes/Proteins in the IL-6$^{high}$ patients. Proteins in the complement system were highly clustered (red nodes) and shared a high number of protein-protein interactions. IL-6, interleukin 6.

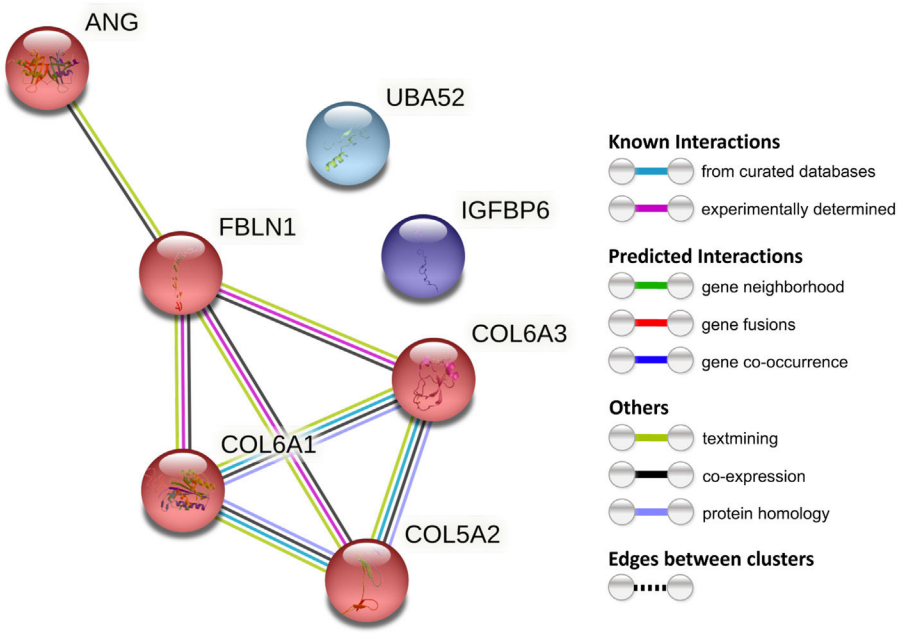


**Fig. 5.**

Analysis of protein-protein interactions by Search Tool for the Retrieval of Interacting Genes/Proteins in the IL-$6^{low}$ patients. Extracellular matrix proteins were clustered (red nodes) and shared a high number of protein-protein interactions. IL-6, interleukin 6.

## Table 1

Proteins upregulated in IL-6$^{high}$ patients compared to IL-6$^{low}$ patients.

| Protein name | Abbreviation | IL-6$^{low}$ fold change | | IL-6$^{high}$ fold change | | *t* test |
|---|---|---|---|---|---|---|
| | | Mean | SD | Mean | SD | *P* value |
| Platelet glycoprotein Ib alpha chain precursor | GPIb (CD42) | 25.47 | 46 | 83.34 | 40.82 | .0294 |
| Cysteine-rich secretory protein 3 isoform 3 | CRISP3 | 12.78 | 35.25 | 75.18 | 49.63 | .0397 |
| Transferrin receptor protein 1 isoform 1 | TFRC | 0.94 | 0.92 | 75 | 49.99 | .0297 |
| Protein Z-dependent protease inhibitor isoform X1 | SERPINA10 | 0.42 | 0.82 | 67.14 | 50.91 | .0237 |
| Biotinidase isoform 1 precursor | BTD | 14.32 | 37.77 | 66.96 | 51.18 | .0335 |
| Collagen alpha-1(V) chain isoform 1 preproprotein | COL5A1 | 16.6 | 29.73 | 61.92 | 51.15 | .0438 |
| Pregnancy zone protein isoform X1 | PZP | 1.35 | 0.84 | 50.79 | 53.91 | .0373 |
| Collectinr-11 isoform f precursor | CL-11 | 1.17 | 0.85 | 50.23 | 54.52 | .0393 |
| Extracellular matrix protein 1 isoform 3 precursor | ECM1 | 0.86 | 1.07 | 34.04 | 51.1 | .046 |
| Haptoglobin isoform 1 preproprotein | HP | 1.67 | 0.76 | 7.3 | 6.54 | .0443 |
| Hemopexin precursor | HPX | 2.73 | 2.18 | 6.67 | 5.39 | .0495 |
| Complement C5 isoform 2 | C5 | 0.82 | 0.61 | 2.16 | 1.87 | .0414 |
| Complement C1r subcomponent-like protein isoform 1 | C1R | 0.69 | 0.81 | 2.02 | 0.99 | .022 |
| Complement C3 preproprotein | C3 | 0.8 | 0.56 | 1.6 | 0.72 | .0496 |
| Complement C4-B preproprotein | C4B | 0.81 | 0.62 | 1.58 | 0.41 | .0299 |
| Heparin cofactor 2 precursor | SPERPIND1 | 0.85 | 0.36 | 1.57 | 0.8 | .0392 |
| Complement component C8 beta chain isoform 1 | C8B | 0.89 | 0.7 | 1.55 | 0.44 | .0371 |
| Complement C2 isoform 1 preproprotein | C2 | 0.77 | 0.66 | 1.39 | 0.72 | .0434 |
| Complement component C9 preproprotein | C9 | 0.7 | 0.49 | 1.33 | 0.6 | .0282 |

*t* test, $P < .05$.

## Table 2

Proteins downregulated in IL-$6^{high}$ patients compared to IL-$6^{low}$ patients.

| Protein name | Abbreviation | IL-$6^{low}$ fold change | | IL-$6^{high}$ fold change | | t-test |
|---|---|---|---|---|---|---|
| | | Mean | SD | Mean | SD | *P* value |
| Transforming growth factor beta-1 proprotein isoform X1 | TGF-$\beta$1 | 0.81 | 0.67 | 0.1 | 0.23 | .0359 |
| Chondroadherin isoform X1 | CHAD | 0.71 | 0.29 | 0.13 | 0.13 | .0015 |
| Fibroblast growth factor-binding protein 2 | FGFBP2 | 1.15 | 0.74 | 0.17 | 0.19 | .0026 |
| Endothelial protein C receptor precursor | PROCR | 0.77 | 0.42 | 0.18 | 0.38 | .0126 |
| Ferritin light chain isoform X1 | FLT | 0.77 | 0.69 | 0.22 | 0.22 | .0194 |
| Proteoglycan 4 isoform X1 | PRG4 | 0.84 | 0.38 | 0.27 | 0.21 | .0019 |

*t* test, $P < .05$.

**Table 3**

KEGG pathways upregulated in IL-6$^{high}$ patients at 4 weeks after anterior cruciate ligament reconstruction.

| KEGG pathway | Total proteins, % | Count | Proteins | *P* value | Benjamini |
|---|---|---|---|---|---|
| Complement and coagulation cascades | 25.9 | 7 | C2, C3, C4B, C5, C8B, C9, SERPIND1 | .0000000023 | 0.00000011 |
| Systemic lupus erythematosus | 22.2 | 6 | C2, C3, C4B, C5, C8B, C9 | .0000045 | 0.00011 |
| Staphylococcus aureus infection | 14.8 | 4 | C2, C3, C4B, C5 | .00016 | 0.0025 |
| Pertussis | 14.8 | 4 | C2, C3, C4B, C5 | .00042 | 0.005 |
| Amoebiasis | 14.8 | 4 | COL5A1, C8B, C9, TGFB1 | .0011 | 0.011 |
| Prion disease | 11.1 | 3 | C5, C8B, C9 | .0021 | 0.017 |
| Phagosome | 14.8 | 4 | COMP, COLEC11, C3, TFRC | .0031 | 0.021 |
| ECM-receptor interaction | 11.1 | 3 | COMP, COL5A1, GP1BA | .013 | 0.078 |

Abbreviation: KEGG, Kyoto Encyclopedia of Genes and Genetics.

**Table 4**

Proteins upregulated in IL-$6^{low}$ patients compared to IL-$6^{high}$ patients.

| Protein name | Abbreviation | IL-$6^{low}$ fold change | | IL-$6^{high}$ fold change | | t-test |
|---|---|---|---|---|---|---|
| | | Mean | SD | Mean | SD | *P* value |
| Insulin-like growth factor-binding protein 6 precursor | IGFBP-6 | 58.58 | 51.67 | 0.28 | 0.27 | .0243 |
| Collagen alpha-1(VI) chain precursor | COL6A1 | 41.17 | 50.63 | 1.67 | 1.64 | .0358 |
| Ubiquitin-60S ribosomal protein L40 isoform X1 | UBA52 | 37.61 | 51.67 | 0.01 | 0 | .0393 |
| Fibulin-1 isoform D precursor | FBLN1 | 34.08 | 49.45 | 0.43 | 0.49 | .0337 |
| Angiogenin precursor | ANG | 33.62 | 42.37 | 0.35 | 0.59 | .0304 |
| Collagen alpha-2(V) chain preproprotein | COL5A2 | 14.80 | 30.15 | 2.65 | 1.96 | .0388 |
| Collagen alpha-3(VI) chain isoform 1 precursor | COL6A3 | 3.15 | 1.95 | 1.61 | 1.23 | 0.0366 |

*t* test, $P < .05$.

**Table 5**

KEGG pathways upregulated in IL-6$^{low}$ patients at 4 weeks after anterior cruciate ligament reconstruction.

| KEGG pathway | Total proteins, % | Count | Proteins | *P* value | Benjamini |
|---|---|---|---|---|---|
| ECM-receptor interaction | 42.9 | 3 | COL5A2, COL6A1, COL6A3 | .00047 | 0.0017 |
| Protein digestions and absorption | 42.9 | 3 | COL5A2, COL6A1, COL6A3 | .00048 | 0.0017 |
| Focal adhesion | 42.9 | 3 | COL5A2, COL6A1, COL6A3 | .0026 | 0.0061 |
| PI3K-Akt signaling | 42.9 | 3 | COL5A2, COL6A1, COL6A3 | .0073 | 0.013 |

Abbreviation: KEGG, Kyoto Encyclopedia of Genes and Genetics.