Proteomic analysis of human synovial fluid reveals potential diagnostic biomarkers for ankylosing spondylitis

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Research

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Abstract

Background Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease affecting the axial skeleton and peripheral joints. The etiology of this disease remains poorly understood, but interactions between genetic and environmental factors have been implicated. The present study identified differentially expressed proteins in the synovial fluid (SF) of AS patients to elucidate the underlying cause of AS.

Methods A cohort of 40 SF samples from 10 AS and 10 each of rheumatoid arthritis (RA), gout, and osteoarthritis (OA) patients were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) to identify differentially expressed proteins specific to AS. The label-free LC-MS/MS results were verified by western blotting. Results We identified 8 proteins that were >1.5-fold upregulated in the SF of AS patients compared to that of the disease control groups, including HP, MMP1, MMP3, serum amyloid P-component (APCS), complement factor H-related protein 5 (CFHR5), mannose-binding lectin 2 (MBL2), complement component C9 (C9), and complement C4-A (C4A). CFHR5 and C9 were previously found in serum from AS patients, while APCS was previously found in SF as well as in serum. However, the present study has identified C4A, and MBL2 as potential AS biomarkers for the first time. The expression levels of MMP3, C9, and CFHR5 were verified in AS SF using western blotting.

Conclusion We performed quantitative comparative proteomic analysis using by LC-MS/MS of the SF from four disease states: RA, gout, and OA. This systematic comparison revealed novel differentially expressed proteins in AS SF, as well as two previously reported candidate biomarkers. We further verified the expression of MMP3, C9 and CFHR5 by western blot. These proteins may serve as diagnostic or prognostic biomarkers in patients with AS, and may thus improve the clinical outcomes of this serious disease.

Background

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease, affecting the axial skeleton and peripheral joints, that occurs in 0.5% of the general population [1]. Without proper clinical treatment, AS causes permanent structural changes, leading to progressive disability that affects the quality of life [2]. While the exact etiology of this disease remains poorly understood, it may be caused by interactions between genetic and environmental factors [3]. AS represents an increasing financial burden on both patients and the healthcare system [4, 5].

AS is defined by the presence of definite structural changes in the sacroiliac joints. Although several possibilities for the etiology of AS, including human leukocyte antigen (HLA)-B27 and tumor necrosis factor-alpha (TNF-α), have been proposed as a therapeutic target [2, 4, 5], no clear cause for AS has been identified. Peripheral arthritis in the knees develops during the early disease course of AS, which can be a prognostic indicator for this disease [5, 6]. Swelling of the knees with inflammatory pain is a

representative symptom of peripheral arthritis in early phase AS patients, and the synovial fluid (SF) from the knees may therefore contain diagnostic biomarkers for AS disease.

SF lubricates the joints, and consists of hyaluronic acid, inflammatory cells, and secreted proteins from the synovial fibroblasts, synovial membranes, and inflammatory cells [7]. Normally, SF is present in small amounts in all joints, acting as joint lubricant and providing nutrition for articular cartilage. However, when inflammation occurs, synovial cells secrete a large amount of synovial fluid and become the subjects of an inflammatory reaction that destroys the joints. Arthrocentesis is the extraction of synovial fluid and has been used for differential diagnosis in patients with inflammatory arthritis [8]. Although serologic and imaging techniques have been developed to diagnose rheumatic diseases, synovial fluid analysis is still used as an important diagnostic tool for differential diagnosis of arthritis, especially acute arthritic disease, since SF is in contact with the primary tissues affected by arthritic diseases and is implicated in the disease pathophysiology. Therefore, it is an excellent biofluid for the discovery of candidate biomarkers in arthritis-related diseases like AS [9].

In the last decade, many proteomic studies in the area of rheumatic diseases have been published [10–14], largely with the aim of diagnosing disease and evaluating disease activity/severity and therapeutic response [11–12]. Different rheumatic disease samples with complicated pathologic structure, such as blood, SF, synovial tissue, and urine, have been investigated for system-wide discovery and validation of rheumatoid arthritis biomarkers [15]. Currently used diagnostic tests for rheumatoid arthritis (RA) include rheumatoid factor and anti-CCP antibodies (anti-cyclic citrullinated peptide antibodies, anti-CCP Ab) [16]. However, there is still no clinically available protein biomarker for early diagnosis and monitoring of AS. Although HLA-B27 is currently used for AS diagnosis, it has a high incidence of false positives. Although there were differences by race, the frequency of HLA-B27 was 4% in the normal control group and 83.3% in the patients with ankylosing spondylitis. Patients with negative HLA-B27 tend to be delayed in diagnosis because the symptoms are not typical. Therefore, the discovery of new robust biomarkers for AS is required for effective early diagnosis and treatment. In the present study, we performed a quantitative proteomics comparison of SF proteins isolated from AS patients [17] and three other arthritis patient groups (RA, gout, and OA). OA is a non-inflammatory arthritis, and was included as a disease control for inflammatory arthritic diseases [18], while both RA and gout were disease controls for inflammatory arthritis from different pathologic origins [19]. Following immunodepletion of super-abundant proteins and label-free quantitative proteomics, we found eight AS-specific and differentially expressed proteins in the AS group compared to the disease control groups. Further western blot verification confirmed the discriminatory ability of seven of these proteins in AS patients. Among these, MMP3 (Matrix metalloproteinase-1), C9 (complement component C9) and CFHR5(Complement factor H-related protein 5) are upregulated in the SF of AS patients, and could therefore be potential biomarkers for AS diagnosis.

Methods

Subjects

Ten patients with AS were recruited from an outpatient rheumatology clinic in Hanyang University Hospital for Rheumatic Diseases, Seoul, South Korea. An additional 30 patients treated at Keimyung University Dongsan Hospital, Daegu, South Korea were recruited as disease control groups (10 OA, 10 gout, and 10 RA). The patients met the 1984 New York criteria for ankylosing spondylitis [17] and the American College of Rheumatology criteria for RA [16], knee OA [18], and acute gout [19]. We collected demographic and clinical data from the subjects, including age, gender, disease duration, blood chemistry, and concomitant treatment. Table 1 shows the diagnosis, clinical data, and current treatments of each patient enrolled in this study. The samples were obtained after getting informed consent from the patients. The study was approved by the ethical committee of the Keimyung University Dongsan Hospital (IRB 2015-12-022).

Synovial fluid sample collection and processing

Synovial fluid was collected by arthroplasty in patients with knee joint pain and swelling. Contaminated bloods during arthroplasty and samples where synovial fluids were generated for reasons other than the respective diseases were excluded. After centrifugation at 500 x *g* for 10 min, five 1 mL vials of supernatant and 1 vial of sediment from each sample were stored at -80 °C through the Keimyung University Dongsan Hospital Human Resource Bank.

Immunodepletion of abundant proteins with MARS cartridge

We used a MARS spin cartridge (Agilent; Santa Clara, CA, USA) to remove highly abundant proteins (albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin), which occupy about 70% of the total SF proteome. The 0.22 μm membrane filter (Agilent; Santa Clara, CA, USA) was used to remove particulates from the fluid samples by centrifugation at 100 x *g* for 1.5 min. The flow-through was mixed with Buffer A LOAD/WASH (Agilent; Santa Clara, CA, USA) and depleted according to the manufacturer's instructions (Agilent Technologies, USA). During the depletion, flow-through was collected, and protein concentration was determined using a BCA assay. The column was routinely regenerated by eluting bound high-abundance proteins with buffer B and neutralizing with buffer A before further use. The acquired proteins were directly digested for total proteomic analysis.

Peptide sample preparation

In-solution tryptic digestion and peptide cleanup were simultaneously performed in a 96-well plate for high reproducibility. Each depleted sample was supplemented with 8 M Urea in 100 mM ammonium bicarbonate (Sigma, St. Louis, MO, USA) and incubated for 20 min at room temperature. The samples were homogenized by vortexing and sonication thrice. To each sample, DTT was added to be 10 mM for

protein reduction at RT for 1 hr. Then, IAA was added to be 30 mM for cysteine bond alkylation in the dark at RT for 30 min. Samples were then diluted with 100 mM ABC prior to the addition of trypsin at a 1:50 trypsin: sample ratio, and incubated at 37℃ overnight. The trypsin was inactivated by acidification with 0.4% TFA. The acidified digests were immediately processed using a Sep-Pak C18 96-well plate (100 mg C18 sorbent per well, Waters). The peptides were eluted with 80% acetonitrile (ACN) and then dried in a vacuum centrifuge.

LC-MS/MS experiments

We performed a comprehensive label free quantitative proteomics analysis using LC-MS/MS to identify proteins in synovial fluid. LC–MS/MS analysis of 40 peptide samples was carried out on an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to a nanoACQUITY UPLC (Waters, Milford, MA) with an in-house-packed trap (150 μm i.d. × 3 cm) and analytical column (75 μm i.d. × 100 cm) using 3 μm Jupiter C18 particles (Phenomenex). The LC gradient was as follows: from 5% to 40% solvent B (acetonitrile with 0.1% formic acid) for 130 min, then 40% to 80% solvent B for 5 min, holding at 80% solvent B for 10 min, and then equilibrating at 95% solvent A (water with 0.1% formic acid) for 30 min. Full MS data were acquired in a scan range of 375–1,575 Th at a resolution of 60,000 at m/z 200, with an automated gain control (AGC) target value of 4.0×10^5 and a maximum ion injection time of 50 ms. The maximal ion injection time for MS/MS was 50 ms at a resolution of 15,000 and an AGC target value of 5×10^4. The dynamic exclusion time was set to 30 s. The resulting 40 .raw files were analyzed using MaxLFQ in Maxquant software (Figure 1).

Data analysis

MaxQuant (v. 1.5.1.2) [20] was used to compare acquired spectra to the Uniprot human database (obtained in June 2018). Carbamidomethylation of cysteine was selected as a fixed modification and N-acetylation and oxidation of methionine were set as variable modifications. A false discovery rate (FDR) cutoff of 1% was applied at the peptide spectrum match (PSM) and protein levels. An initial precursor mass deviation of up to 4.5 ppm and a fragment mass deviation of up to 20 ppm were allowed. Protein identification required at least one peptide using the ‘razor plus unique peptides’ setting. Proteins were quantified using the XIC-based label-free quantification (LFQ) algorithm in MaxQuant [21]. The ‘match between runs’ option was used for nonlinear retention time alignment. The match time window was 0.7 min, and the alignment time window was 20 min. Further statistical and bioinformatics analysis was performed using Perseus software (v. 1.5.3.2). Proteins representing hits to the reverse database, contaminants, and proteins only identified by sites were eliminated prior to analysis. A minimum of three valid values across each clinical group was required for each protein group for quantification. The LFQ intensity for each protein group was compared between clinical groups following log2 transformation. Statistical analysis of the log2-transformed data was performed using two samples t-test (p value <

0.05). Proteins with ≥ 1.5 fold change between AS and other patient groups were considered to be differentially expressed proteins (DEPs) for AS group. The resulting DEPs were submitted to gene ontology annotation enrichment analysis.

Enrichment and network analysis

To explore functional enrichment in the identified proteins in each of the clinical groups, GO analysis using DAVID (version 6.8) [22] was performed. We identified biological processes and KEGG pathways [23] that were enriched in our dataset with p values less than 0.05 [22]. To construct a network describing the enriched GO terms, we selected DEPs that are involved in enriched cellular processes. We then built a protein network model using protein interaction information obtained from STRING version 11 database [24]. The interaction network models were visualized using Cytoscape [25].

Western blot verification

We verified the results of our LC-MS/MS analysis using western blotting to confirm the abundance of selected proteins in the SF of patients from each of the clinical groups. SF was clarified by incubation with hyaluronidase (Sigma, H3884) at room temperature for 10 min, and then diluted 1:10 in RIPA buffer (ThermoFisher, Waltham, MA) containing protease inhibitor (Roche Diagnostics GmbH, Mannheim) and phosphatase inhibitor (Roche Diagnostics GmbH, Mannheim, MA). The diluted sample was incubated on ice for five minutes, then transferred into new tubes and centrifuged at 10000 x *g* at 4°C for 15 min. Equal amounts of protein (30 ug) in each sample were aliquoted following protein quantitation by BCA assay (BCA Protein Assay, ThermoFisher, Waltham, MA). Samples were mixed with sodium dodecyl sulfate (SDS) loading buffer and separated using SDS polyacrylamide gel electrophoresis (Western Blotting Kit, Hoefer Inc., Holliston, MA). Proteins were then transferred onto nitrocellulose membranes (Amersham, Chicago, IL, USA), blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and immunoblotted with the appropriate primary and secondary antibodies. The antibodies used were as follows: rabbit polyclonal antibody for C9 (1:5000, PA5-29093, ThermoFisher, Waltham, MA), CFHR5 (1:500, ab175254, Abcam, Cambridge), MMP3 (1:5000, ab52915, Abcam, Cambridge, MA), Peroxidase-conjugated AffiniPure Donkey Anti-Rabbit IgG (H+L) (1:10,000, Jackson Immunoresearch, Baltimore Pike, West Grove, PA).

Results

General characteristics of study subjects

The general characteristics of the study subjects are presented in Table 1. The AS group was 32.6 (±10.6) years of age, with 7.0 (± 4.6) years mean disease duration, 80% HLA-B27 positive rate, 20% biologics utilization, 59.5 (±39.7) mm/hr averaged ESR, and 4.9 (±4.3) mg/dL averaged CRP. The RA group were all

females at 59.6 (± 11.6) years of age, with 4.9 (± 4.9) years mean disease duration, 90% rheumatoid factor and anti-CCP antibody positive rate, 30% biologics utilization, 32.7 (± 17.0) mm/hr averaged ESR, and 0.5 (± 3.7) mg/dL averaged CRP. The gout group were all males at 62.8 (± 12.8) years of age, with 0.3 (± 0.9) years of disease duration, and 7.1 (± 2.6) mg/dL concentration of serum uric acid. The OA group were 64.8 (± 8.5) years of age, with 5.5 (± 5.9) years mean disease duration.

Proteomic analysis of SF from AS, RA, gout, and OA patient groups

To obtain insight into the molecular basis of AS in the SF, we performed a label-free quantitation (LFQ)-based proteomic comparison of SF from AS patients and SF from patients with OA, gout, and RA (Figure 1a). All synovial fluids were first subjected to immunodepletion of the 14 most abundant proteins using an IgY-14 depletion kit. Tryptic peptides from each sample were analyzed on an Orbitrap Fusion Lumos mass spectrometer, identifying 569 proteins and 6,103 peptides in the AS group, 874 proteins and 9,991 peptides in the RA group, 456 proteins and 5,313 peptides in the OA group, and 873 proteins and 8,231 peptides in the gout group at <1% FDR (Figure 1b). A further MQ analysis using ‘match between runs’ to minimize missing data between replicates and groups [21, 26] was then carried out. To resolve the differentially expressed proteins (DEPs) in AS compared to those in other diseases, LFQ values were compared for proteins with three valid values in each group using Perseus. A total of 385 proteins for AS/OA, 500 proteins for AS/Gout, and 485 proteins for AS/RA were quantified in this manner (Supplementary Table S1). Further refinement for proteins with > 1.5 fold-change resulted the identification of 102 and 84 proteins in AS/OA, 41 and 179 proteins in AS/Gout, and 69 and 151 proteins in AS/RA, for upregulated and downregulated proteins, respectively (Supplementary Table S2). Intriguingly, eight of the DEPs were specifically upregulated in the AS patient group: HP (Haptoglobin), MMP1 (Matrix metalloproteinase-1), MMP3, APCS (Serum amyloid P-component), CFHR5, C9, C4A (Complement C4-A), and MBL2 (Mannose-binding protein C) (Figure 2a and Table 2). Similarly, a total of 24 proteins, including immune-related proteins such as PLA2G2A (Phospholipase A2) and TF (Transferrin), were specifically downregulated (Figure 2c).

Gene ontology analysis to clarify the molecular basis for AS

To gain insight into the functional roles of the DEPs associated with AS (Figure 2a and Table 2), we carried out GO analysis to identify enriched biological process and molecular function GO terms using the up- and downregulated DEPs (Supplementary Tables S3 and S4). Interestingly, the specifically upregulated proteins in AS were enriched for immune response-related biological processes (Figure 2b). The proteins that are specifically upregulated in AS compared to OA and gout were associated with the innate immune response (Figure 2b). The enriched GO terms suggested that the specifically upregulated proteins play an important role in complement activation and the inflammatory response. In addition, GO terms involved in metabolic processes, protein oligomerization, proteolysis, and extracellular matrix

disassembly were enriched in the AS-specific proteins. Conversely, proteins that were downregulated specifically in the AS group were enriched for GO terms relating to biological processes including extracellular matrix organization, collagen fibril organization, and cellular structure (Figure 2c).

Protein-protein interaction network describing AS

To better understand the cellular networks that are altered in the synovial fluid of AS patients, we created protein-protein interaction network models of AS-specific upregulated DEPs using the STRING database (Figure. 3). Four important processes were enriched in the AS-specific upregulated DEPs: innate immune response, complement activation, platelet degranulation, and glycolytic process. We found that APCS (innate immune response), C9, C4A, CFHR5, and MBL2 (complement activation), which are common in AS SF, are known to interact based on this network.

Verification by western blot analysis

Following the LC-MS/MS analysis, the levels of 7 of the DEPs (MMP3, CFHR5, C9, MBL2, C4A, APCS, and MMP1), except for HP, which occupy a large amount in synovial fluid, were verified in SF by western blotting with specific primary antibodies (Figure 4). MMP3 was used as a positive control because it is known to be highly expressed in the SF of AS patients [27]. As expected, the expression levels of MMP3 (50 kDa) were significantly increased in AS SF compared to that in the other clinical groups. CFHR5 and C9 had higher expression levels in AS than the control. The expression levels of the remaining proteins could not be verified by western blotting.

Discussion

Early diagnosis of AS is difficult because the etiology is not clear and there is no specific diagnostic indicator [28]. Mass spectrometry-based proteomics is a state-of-the-art analytical technique that enables the discovery of indicator proteins for the diagnosis and treatment of diseases. There has been a recent expansion in proteomics research on a number of different rheumatic diseases [29].

In the present study, we performed comprehensive proteomic profiling and western blot analysis of SF obtained from patients with AS and SF obtained from patients with comparative diseases (RA, gout, and OA). SF samples obtained from patients with AS and the three disease control groups were quantified using highly sensitive LC-MS/MS and LFQ-based analysis. We discovered eight biomarker candidate proteins (HP, MMP3, CFHR5, C9, MBL2, C4A, APCS, MMP1) with a fold change > 1.5 in AS compared to that in the other groups. Among these, the expression of seven proteins (with the exception of HP) that were abundant in SF was verified by western blot analysis. MMP3, CFHR5, and C9 were highly expressed in the SF of patients with AS.

MMP family proteins are involved in the pathogenesis of arthritis. In particular, MMP3 is a protease that is synthesized and secreted by fibroblasts and chondrocytes in synovial joints, and also activates other MMPs such as MMP1, MMP7, and MMP9 [30]. A recent meta-analysis suggests that the serum levels of MMP3 rise in AS patients [27]. The validity of our results was further enhanced by the discovery of previously researched AS relevant protein, MMP3.

CFHR5 plays an important role in the alternative pathway complement system, and binds to C3 to activate it [31]. This protein is highly expressed in the serum of AS patients [32]. In addition, high concentrations of CFHR5 were observed in the plasma of patients with systemic lupus erythematosus, an autoimmune disease [33]. C9 is a member of the membrane attack complex (MAC) complement system, and causes lysis by inducing pores in the cell membrane after activating C5, the final stage of the complement system [34]. The levels of this protein are elevated in the serum of AS patients [32].

According to the proteomic analysis, MBL2 and C4A were dysregulated in the AS group compared to in the other disease groups, but there was no detectable difference in the level of this protein according to the western blotting results. The expression of MBL2 (26 kDa) was observed in 7 out of 10 cases of AS in the western blot, compared to six in RA, four in Gout, and one in degenerative arthritis. C4A (95 kDa) was expressed in all 10 cases of AS but had a similar level of expression in the other disease groups. Both of these proteins are complementary proteins, and MBL2 has the ability to recognize carbohydrate types found on pathogenic microbial surfaces. MBL2 initiates the lectin pathway and opsonizes apoptotic and necrotic cells [35]. C4A is involved in the classical pathway of the complement system. Deficiency of C4A is associated with systemic lupus erythematosus and type 1 diabetes mellitus, and its overexpression is associated with mental disorders such as schizophrenia and bipolar disorder [36]. The complement system is an important mechanism of humoral and innate immunity. Suppressing the complement system in an animal model of AS may improve AS treatment [37]. The complement system is active in patients with systemic sclerosis, a musculoskeletal disorder [38].

APCS (25 kDa) was observed in all disease groups by western blot. APCS is associated with the innate immune system and has increased expression in the SF and serum of AS patients [32, 39]. MS-based proteomic analysis revealed that APCS had a higher fold-change in the AS group than the other groups (AS/RA: 15.69, AS /Gout: 4.81, AS/Degenerative Arthritis: 6.53) (Table 2), but this could not be verified by western blotting. In one study, CRP and APCS were shown to have 51% sequence homology in rats [40]. The antibody used in the present study may have therefore been detecting an inflammatory marker that may have little specificity when it comes to AS. This antibody-based approach may therefore not be suitable in all cases, and an alternative approach may be better. Proteins that have not been verified in the present study by western blotting also need to be verified in future studies, and multiplexed verification using targeted proteomics may provide more specific quantitative measurements than western blotting. MMP1 (50 kDa), a protein mainly expressed in bone metabolism, which was expected to have high levels in SF from AS patients, was not detected in all disease groups, and should be studied further.

The limitations of the present study are the following: the relatively small number of patients included due to the difficulty in obtaining clinical sample donor and the lack of further validation beyond western blotting. Further validation will increase the likelihood of identifying biomarkers for AS, as will increasing the number of samples and using alternative sample types including urine, serum, synovial membrane, and animal models.

Conclusions

In total, 1,089 proteins were identified by label-free comparative proteomic analysis in the SF of patients with AS, RA, gout, and OA. This is the largest dataset of proteins identified in the synovial fluid to date. This is also the first time that three diseases (RA, gout, and OA) have been used simultaneously as disease control groups during proteomic profiling of AS. Out of the significantly dysregulated proteins, eight were significantly increased in the SF of AS patients compared to that of patients of the other three diseases. Out of these, MBL2 and C4A were the first proteins reported in AS, and the remaining proteins were first studied in SF. Four out of these eight proteins are part of the complement system, which appears to be highly associated with AS. Altogether, these results suggest an important role for complement signaling during AS disease progression, and this avenue of enquiry may provide insight into the molecular mechanisms underlying AS. The clinical utility of the putative biomarkers identified in the present study should now be further validated in a larger cohort.

Abbreviations

AS: Ankylosing spondylitis, SF: Synovial fluid, OA: Osteoarthritis , RA: Rheumatoid arthritis, HLA: human leukocyte antigen, TNF: tumor necrosis factor, CCP: cyclic citrullinated peptide, ACN: acetonitrile, AGC: automated gain control, FDR: false discovery rate, PSM: peptide spectrum match, LFQ: label-free quantification, DEP: differentially expressed proteins, SDS: sodium dodecyl sulfate, bDMARD: biologic disease-modifying anti-rheumatic drug; cDMARD: conventional disease-modifying anti-rheumatic drug; CRP: C-reactive protein; ETN: etanercept; ESR: erythrocyte sedimentation rate, HP: Haptoglobin, MMP1: Matrix metalloproteinase-1, MMP3: Matrix metalloproteinase-1, APCS: Serum amyloid P-component, CFHR5: Complement factor H-related protein 5, C9: Complement component C9, C4A: Complement C4-A, MBL2: Mannose-binding protein C, MAC: membrane attack complex

Declarations

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Authors' contributions

CNS and JSK conceived and designed the study. JHL and JHJ performed experiments with a support of JK and JR. JHJ, JK, KPK and JSK performed proteomics data analysis. JHL, SHK, WKB, THK, and CNS clinically interpreted the results. CNS, JSK, KPK, JHL, JHJ, and JSR wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All of the raw data has been deposited into the PRIDE Archive (ProteomeXchange) with the data set identifier PXD016620.

Ethics approval

The study was approved by the ethical committee of the Keimyung University Dongsan Hospital (IRB 2015-12-022).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. The clinical and laboratory features of patients with ankylosing spondylitis, rheumatoid arthritis, gout, and osteoarthritis.

Diseases/Number	Gender	Age	ESR (mm/hr)	CRP (mg/dL)	HLA-B27	RF (ng/mL)	Anti-CCP (U/mL)	Serum UA (mg/dL)	Disease Duration (yr)	bDMARD	cDMARD
A1	M	39	81	5.1	Positive	NA	NA	NA	2	IFX	None
A2	M	41	127	15.8	Positive	NA	NA	NA	10	GLM	None
A3	M	47	46	4.8	Negative	NA	NA	NA	3	None	MTX+SSZ
A4	M	21	7	8	Positive	NA	NA	NA	6	None	None
A5	M	24	53	4.7	Positive	NA	NA	NA	7	None	SSZ
A6	M	35	27	2.6	Positive	NA	NA	NA	17	None	MTX+SSZ
A7	M	34	79	2	Positive	NA	NA	NA	5	None	SSZ
A8	M	45	114	2.3	Positive	NA	NA	NA	11	None	SSZ
A9	M	22	39	3.2	Positive	NA	NA	NA	9	None	SSZ
A10	F	18	22	0.74	Negative	9.3	0	6.1	0	None	None
R1	F	57	64	1.4	NA	47.4	>200	6.2	0.3	None	MTX+SSZ
R2	F	47	25	1.04	NA	92	>200	3.1	0.5	None	MTX+SSZ
R3	F	42	45	0.46	NA	39.5	>200	NA	5	None	MTX
R4	F	51	2	0.01	NA	785.4	>200	NA	3	TCZ	MTX
R5	F	69	47	0.04	NA	35.7	129.5	NA	5	IFX	MTX
R6	F	62	26	0.6	NA	310	>200	NA	5	None	MTX+LEF
R7	F	53	39	0.54	NA	46.3	>200	NA	8	None	MTX
R8	F	64	33	0.08	NA	99.7	16.6	5.8	17	None	HCQ
R9	F	76	20	0.85	NA	9.3	0	NA	1	None	MTX

R10	F	75	26	0.02	NA	133	>200	NA	4	ETN	MTX
G1	M	73	NA	0.47	NA	NA	NA	8.4	0	None	None
G2	M	71	105	1.75	NA	9.3	0.6	9	0	None	None
G3	M	47	NA	2.31	NA	NA	NA	7.5	0	None	None
G4	M	62	68	1.6	NA	NA	NA	8.6	0	None	None
G5	M	42	66	4.53	NA	22	1.9	6.9	0	None	None
G6	M	63	NA	NA	NA	9.3	NA	9.9	0.2	None	None
G7	M	51	NA	NA	NA	NA	NA	6.8	0.1	None	None
G8	M	63	NA	NA	NA	NA	NA	9	3	None	None
G9	M	82	54	1.9	NA	11.4	NA	3.5	0	None	None
G10	M	74	52	11.44	NA	9.3	0	1.5	0	None	None
O1	F	70	30	0.06	NA	9.3	NA	5.4	4	None	None
O2	F	53	NA	NA	NA	9.3	0.6	NA	6	None	None
O3	F	64	48	0.1	NA	NA	NA	3.4	4	None	None
O4	F	72	17	0.13	NA	10.5	0	4.1	0	None	None
O5	F	63	NA	NA	NA	9.3	1.1	NA	2	None	None
O6	M	64	NA	NA	NA	9.3	NA	NA	6	None	None
O7	F	57	NA	NA	NA	NA	NA	NA	17	None	None
O8	F	72	NA	NA	NA	NA	NA	NA	1	None	None
O9	F	79	15	0.03	NA	9.3	0.9	7.3	0	None	None
O10	F	54	NA	NA	NA	NA	NA	NA	15	None	None

A: ankylosing spondylitis; Anti-CCP: anti-cyclic citrullinated peptide antibodies; bDMARD: biologic disease-modifying anti-rheumatic drug; cDMARD: conventional disease-modifying anti-rheumatic drug; CRP: C-reactive protein; ETN: etanercept; ESR: erythrocyte sedimentation rate; F: female; G: gout; GLM: golimumab; HCQ: hydroxychloroquine; IFX: infliximab; LEF: leflunomide; M: male; MTX: methotrexate; NA: not available; O: osteoarthritis; R: rheumatoid arthritis; RF: rheumatoid factor; SSZ: sulfasalazine; TCZ: tocilizumab; UA: uric ac

Table 2. List of proteins with increased levels in the synovial fluid of ankylosing spondylitis patients vs other disease groups.

Gene symbol	Protein	Fold change (AS/RA)	Fold change (AS/Gout)	Fold change (AS/OA)
HP	Haptoglobin	2.67	5.93	27.97
MMP3	Matrix metalloproteinase-3	2.02	2.63	10.13
CFHR5	Complement factor H-related protein 5	1.61	4.52	3.23
C9	Complement component C9	1.85	1.75	1.82
MBL2	Mannose-binding protein C	1.68	1.52	1.61
C4A	Complement C4-A	1.92	2.02	1.63
APCS	Serum amyloid P-component	15.69	4.81	6.53
MMP1	Matrix metalloproteinase-1	1.97	2.75	14.96

AS: ankylosing spondylitis; OA: osteoarthritis; RA: rheumatoid arthritis;

Figures

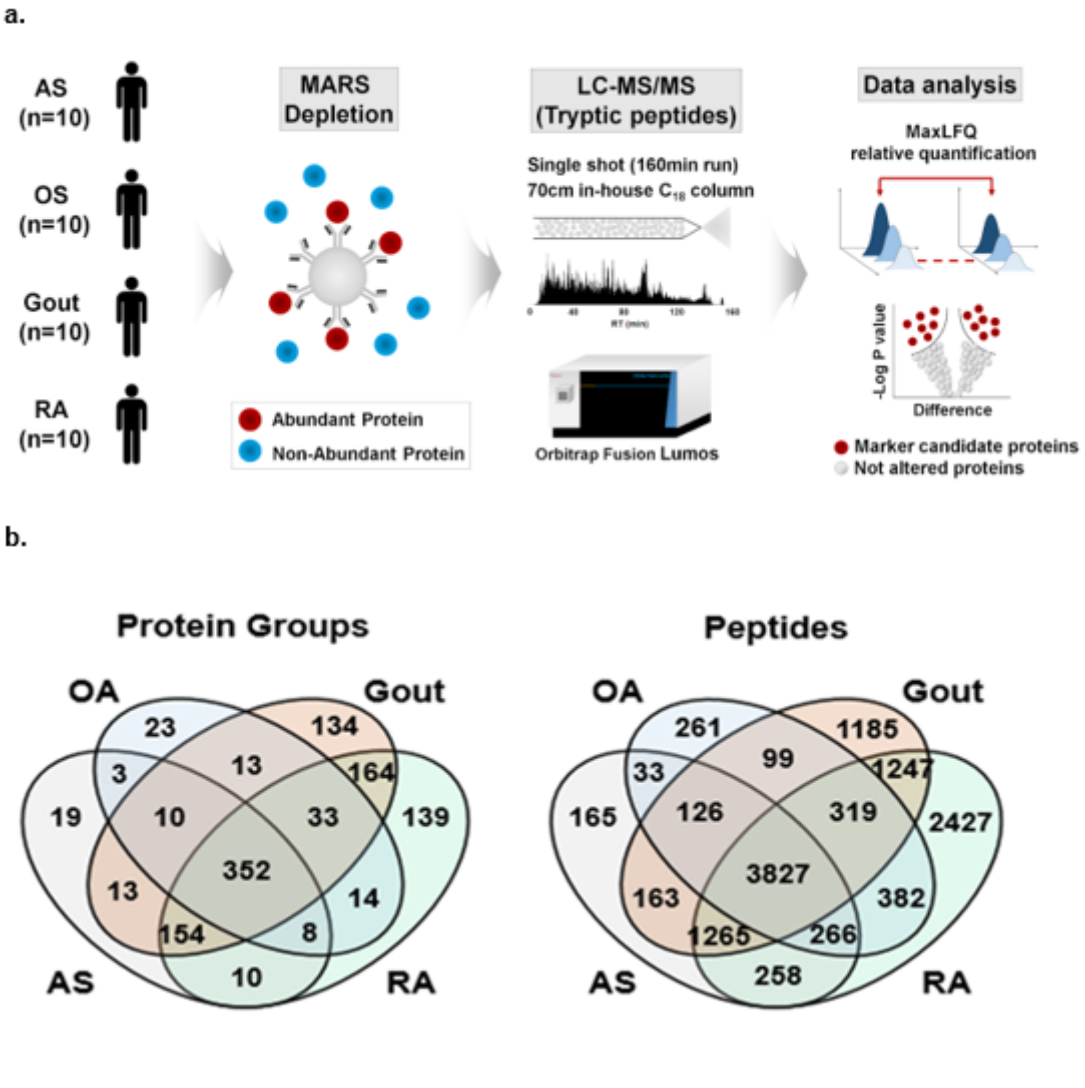

Figure 1

(a) Schematic diagram of proteomics experimental workflow. Synovial fluids of ankylosing spondylitis (AS), osteoarthritis (OA), gout, and rheumatoid arthritis (RA) were subject to immunodepletion using the multiple affinity removal system (MARS) and followed by trypsinization and LC-MS/MS analysis. MaxQuant LFQ (MaxLFQ) analysis was carried out for quantitative comparison. (b) Venn diagram showing the unique and shared protein groups and peptides identified in the synovial fluid of each group.

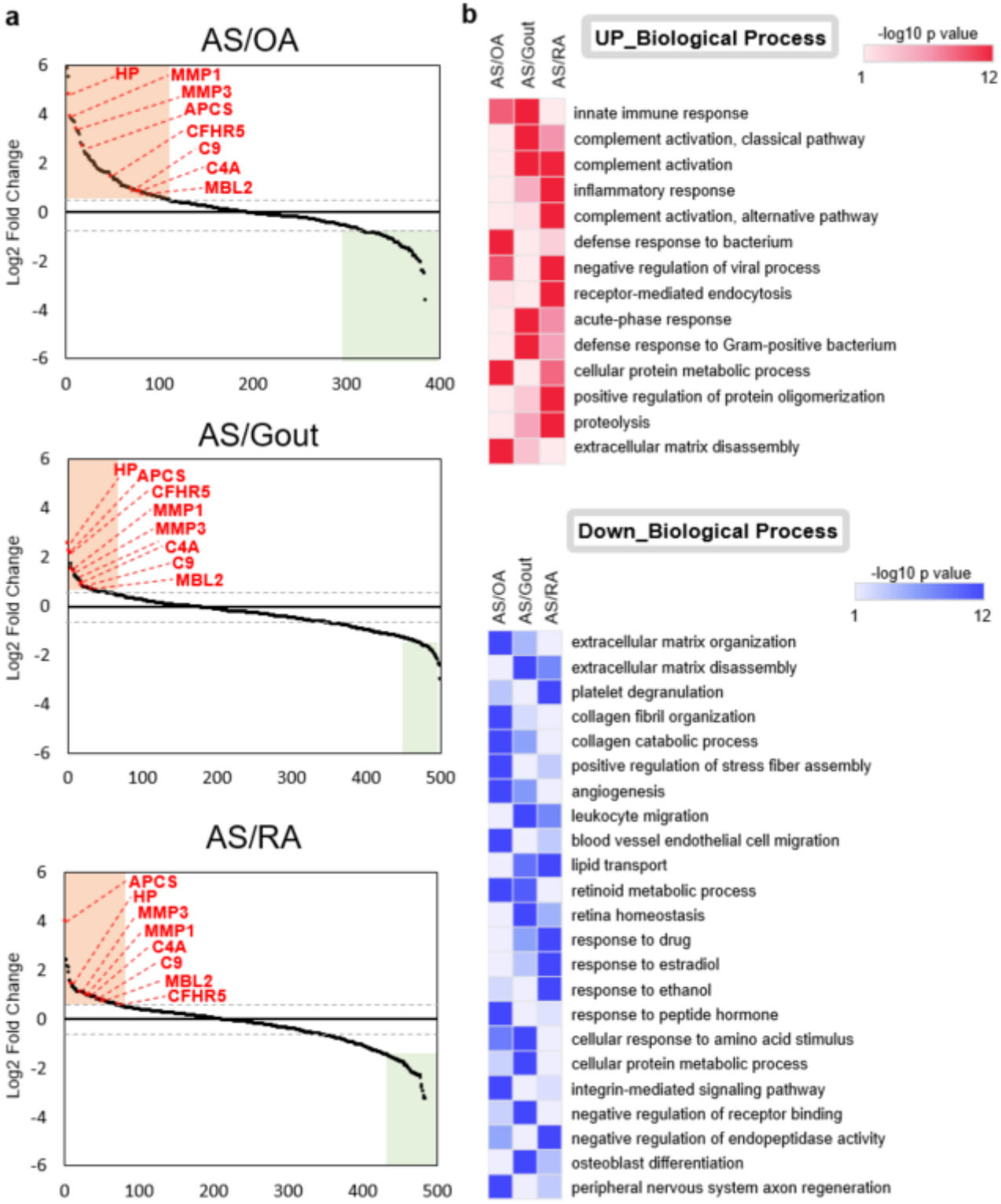

Figure 2

(a) Reverse cumulative plots of protein fold change in the synovial fluid from each disease group. The most commonly upregulated 8 proteins (HP, MMP1, MMP3, APCS, CFHR5, C9, C4A, and MBL2) are highlighted. (b) Gene ontology analysis of differentially expressed proteins in the synovial fluid of the AS group compared to in the OA, gout, and RA groups. Heatmaps showing significantly enriched gene ontology (GO) biological process terms ($p < 0.05$) for differentially expressed proteins. The red color in the

heatmap indicates a significant upregulation and blue color indicates downregulation of the biological process.

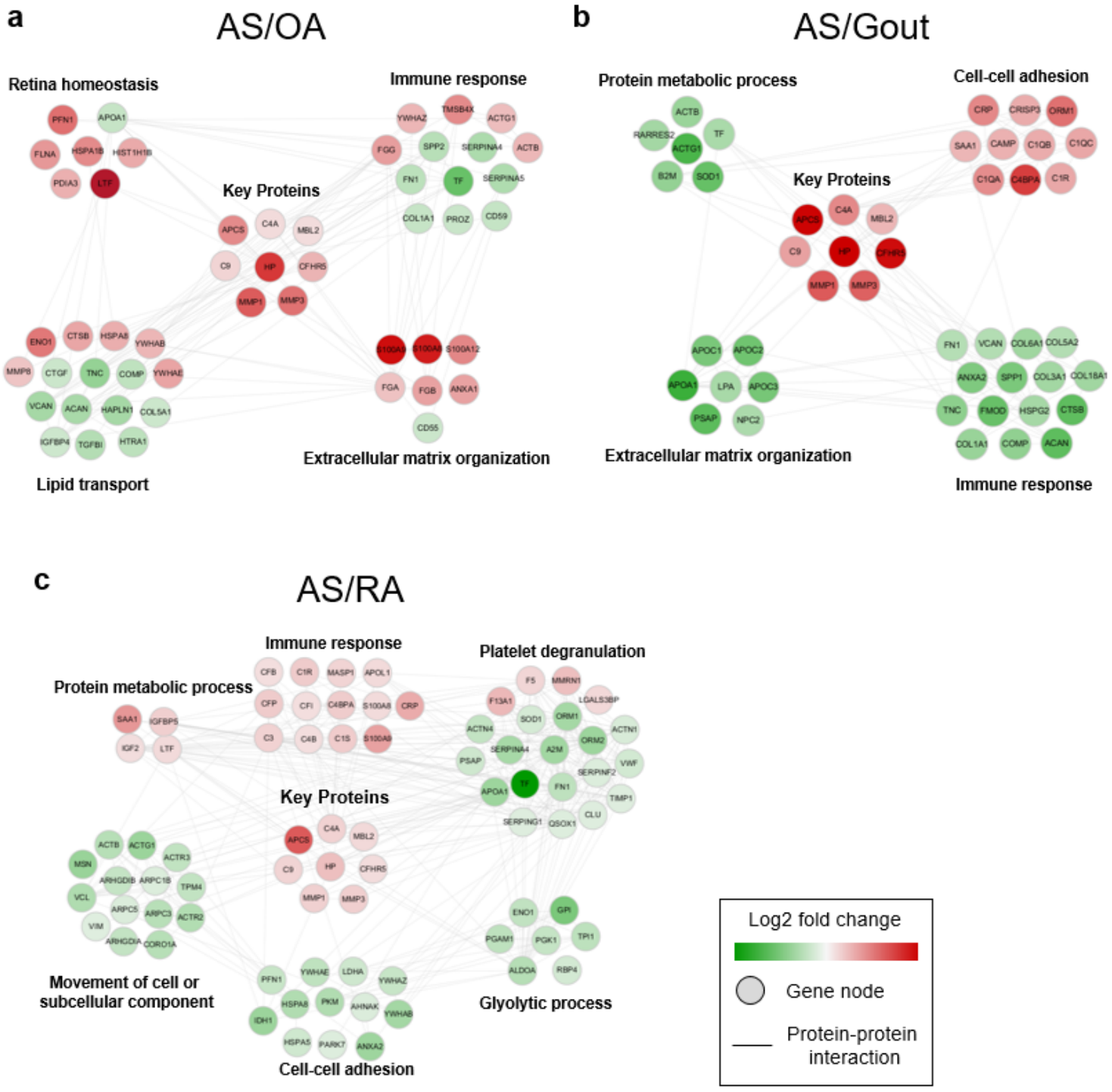

Figure 3

Network modelling of differentially expressed proteins in the synovial fluid of the AS group versus the (a) OA, (b) gout and (c) RA group. Protein-protein interaction network showing the biological processes affected, including the immune response, platelet degranulation, cell-cell adhesion etc. The colors of the nodes represent proteins with increased (red) or decreased (green) evels in the synovial fluid of the AS

group compared to in the other groups. The connection between nodes (solid grey lines) shows either a regulatory role or physical interaction between proteins.

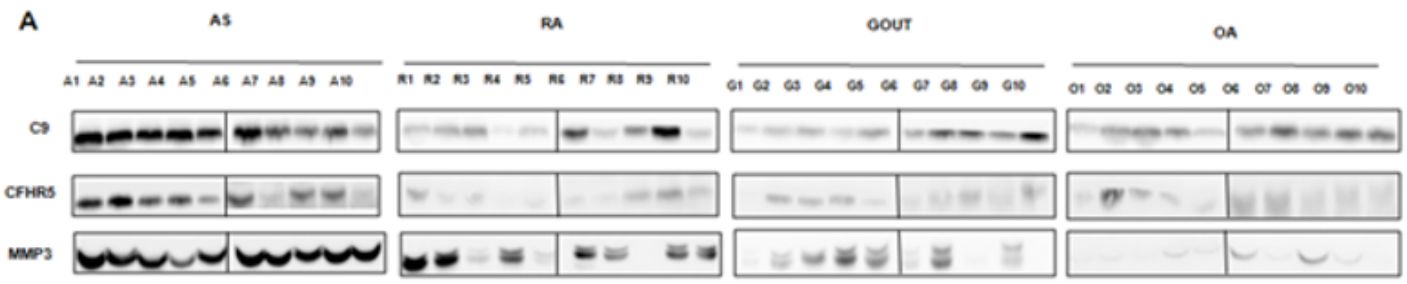

Figure 4

Verification of synovial fluid C9, CFHR5, and MMP3 by western blot. Western blot analysis of C9 and CFHR5 overexpression in AS synovial fluid (n=10) when compared to RA (n=10), gout (n=10), and OA (n=10) synovial fluid (A-AS, R-RA, G-Gout, O-OA).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.Quantifiableproteinlists.xlsx
- SupplementaryTable3.GOBiologicalprocess.xlsx
- SupplementaryTable2.Differentiallyexpressedproteins.xlsx