

# Differential proteomics of the synovial membrane between bilateral and unilateral knee osteoarthritis in surgery-induced rabbit models

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**Abstract.** The present study investigated the differential proteomics of synovial membranes between bilateral and unilateral anterior cruciate ligament transection (ACLT) in rabbits with knee osteoarthritis (KOA), in order to elucidate the pathological biomarkers of different degrees of KOA. A total of 6 New Zealand rabbits were randomly divided into groups A and B (three rabbits per group). The two groups were subjected to bilateral and unilateral ACLT, respectively. A total of 6 weeks following surgery, proteins were extracted from the knee joint synovial membranes of KOA rabbits and were separated by two-dimensional polyacrylamide gel electrophoresis. The differentially expressed proteins in the OA synovial membranes were selected for further analysis by linear ion trap-Fourier transform ion cyclotron resonance mass spectrometry. Ten protein spots were identified to be different between the synovial membranes of the bilateral and unilateral KOA rabbits. Protein disulfide-isomerase and creatine kinase M-type were identified in the unilateral KOA rabbit synovial membranes. Serum albumin (three spots), lumican,  $\alpha$ -2-HS-glycoprotein and three uncharacterized proteins were identified in the synovial membranes of the bilateral KOA rabbits. The differential proteomic expression demonstrated the different biomarkers associated with bilateral and unilateral KOA, and indicated that spontaneous and secondary KOA require diverse methods of treatment; thus the underlying mechanism of KOA requires further investigation.

## Introduction

Osteoarthritis (OA) is one of the most common causes of musculoskeletal disability. It is characterized by progressive degeneration of articular cartilage and synovitis is a significant contributor, which contributes to the development of OA (1). Diagnosis of OA using magnetic resonance imaging indicates synovial hyperplasia proximal to cartilage lesions, particularly in the bursa suprapatellaris and posterior cruciate ligament of the knee (2). A previous study proposed that the synovitis is a significant cause of pain and oedema in OA patients (3). Although interleukin-6 (IL-6), cluster of differentiation 4 (CD4), CD8+ T-cells and adipocytokines (such as adiponectin and leptin) are vital inflammatory factors for the process of synovitis (4,5), the molecular mechanisms of the progressive degeneration of articular synovial membrane in OA remain to be fully elucidated. Previously, alterations in the transcriptomes of OA synovial membranes were investigated using DNA microarray or reverse transcription-quantitative polymerase chain reaction analysis (6,7). However, alterations in mRNA expression levels have been reported to not always correlate well with the protein levels due to post-transcriptional regulation, post-translational modification and differential stability of proteins (8).

The use of proteomics, during which entire proteins in tissues or cells are identified and quantified directly, has been identified as a valuable method for elucidating the molecular basis of disease etiology. Recently, the proteome of human articular chondrocytes, synovial fluid, serum or urine was characterized by two-dimensional polyacrylamide gel electrophoresis (2-DE) and tandem mass spectrometry of cultured chondrocytes isolated from normal cartilage (9-13). However, to the best of our knowledge, few proteomic studies regarding the articular synovial membrane have been conducted (14), and the present study have improved knowledge of the proteome of the synovial membrane, and provided a foundation for further investigation of the pathology of synovial membrane diseases.

Studies regarding the molecular and cellular mechanisms of OA have evaluated bilateral or unilateral joint tissue samples without considering the distinction between spontaneous and

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secondary OA (10,15-18). However, the majority of spontaneous (resulting from the aging process) and secondary (traumatic) knee OA (KOA) cases occur in bi/unilateral knee joints, respectively (19), and the pathological process and treatment for the disease may differ between spontaneous and secondary KOA (20,21). Furthermore, certain studies proposed that proteomics may be important in the treatment of OA (9,22). Thus, the present study hypothesizes that the mechanisms of proteomic alterations in the progressive destruction of articular synovial membrane in spontaneous and secondary KOA are different. The profile of proteins selectively extracted from rabbit synovial membrane samples of bi/unilateral KOA were compared by two-dimensional gel electrophoresis (2-DE) and mass spectral analysis to highlight requirements for establishing diverse treatments for OA resulting from the aging process and traumatic OA.

## Materials and methods

**Animals.** New Zealand White (NZW) rabbits were supplied by the Fujian University of Traditional Chinese Medicine (Fuzhou, China) animal testing center [batch no. SCXK (Shanghai) 2012-0011]. The NZW rabbits, 3 male and 3 female (age, 6 months; weight, 2.5-3.0 kg), were provided with a standard laboratory diet with drinking water and housed in individual cages under a 12-h light/dark cycle at 20-26°C. The present study complied with national legislation and with the Ministry of Health of the People's Republic of China Guide for the Care and Use of Laboratory Animals (23). Local ethical committee approval was obtained for the current study from the ethics committee of the Fujian University of Traditional Chinese Medicine. The NZW rabbits were sacrificed by air embolism 6 weeks following the surgery for OA model induction.

**Animal grouping.** All animals were randomly divided into groups A and B, with 3 rabbits per group, and SPSS 13.0 statistical software was used (SPSS, Inc., Chicago, IL, USA).

**KOA model.** The A and B group rabbits were subjected to bilateral and unilateral anterior cruciate ligament transection (ACLT), respectively. Briefly, the rabbits were administered with intraperitoneal injections of 5% chloral hydrate (3 ml/kg; Qingdao Yulong Algae Co., Ltd., Qingdao, China), in order to sedate and anesthetize them appropriately. The right knee was shaved, sterilized, draped under sterile conditions and a medial arthrotomy was performed. The patella was then dislocated, and the ACL was isolated and transected. ACLT was confirmed by the surgeon and an observer using the Lachman test. Following irrigation using sterile saline solution, the wounds were closed in layers and treated with antiseptic. Rabbits were provided with the appropriate postoperative care and allowed to move freely in individual cages.

**Specimen collection for synovial membrane proteomic detection.** The rabbits were sacrificed 6 weeks subsequent to surgery, and the synovial membrane of the operative right knee joint was dissected in group A and B rabbits. The samples were maintained for subsequent evaluation in a nitrogen canister.

**Protein extraction of the synovial membrane.** Synovial membrane samples were ground into powdered tissue using a pestle and mortar, and transferred into a homogenizer. Lysates (500  $\mu$ l/100 mg) were added to the homogenate; RNase (50  $\mu$ g/ml), DNase (200  $\mu$ g/ml) and 10  $\mu$ l/1 ml lysate were added, and maintained at 4°C for 15 min. The tissue was then centrifuged at 12,000 x g for 60 min at 4°C. The supernatant was collected and the protein concentration was determined using the 2-D Quant kit (GE Healthcare Life Sciences, Uppsala, Sweden) according to the manufacturer's protocols. The tissues were refrigerated at -70°C.

## 2-DE

**Protein solubility and dry strip swelling.** Lysis buffer [500  $\mu$ l; 7 mol/l urea, 2 mol/l thiourea, 4% CHAPS, 1% dithiothreitol (DTT), 0.2% NP-40, 1% ampholine (pH 4-6) and 1% ampholine (pH 3.5-10)] was added into the protein solution extraction, which was then vibrated for 5 h. Dissolution of the protein solution extraction was conducted using a 200 W ultrasonic instrument for 200 sec. The protein solution extraction was centrifuged at 20,000 x g for 20 min at 4°C. The supernatant was collected and the protein concentration was measured using the Bradford Protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The rehydration solution [800  $\mu$ l; 8 mol/l urea, 2% CHAPS, 0.5% ampholine (pH 4-7), 0.002% Bromophenol Blue and 800  $\mu$ g protein solution) was added into the protein electrophoresis tank and the dry strip was immersed (pH 4-7; 18 cm), gum down and incubated room temperature overnight.

**Isoelectric focusing electrophoresis.** The expanded 12% gel was placed into the gel strip slot of the isoelectric focusing electrophoresis apparatus and the gel was covered with covering oil. The electrophoresis parameters were as follows: 500 V for 2 h (gradient); 1,000 V for 1 h (gradient); 8,000 V for 2.5 h (step).

**Gel strip equilibration.** The gel strip was equilibrated twice following isoelectric focusing electrophoresis, for 15 min each time, with gentle agitation. The liquid components for the initial equilibration were as follows: 50 mmol/l Tris-HCl (pH 8.8), 6 mol/l urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS) and 1% DTT. The second equilibrium liquid components were as follows: 50 mmol/l Tris-HCl (pH 8.8), 6 mol/l urea, 30% glycerol, 2% SDS and 2.5% idoacetamide.

**SDS-polyacrylimide gel electrophoresis.** The balance gel was layered on the top of the spacer gel, taking care to avoid trapped bubbles, and the gel strip was fixed with 0.5% agarose. The electrode buffer was added following solidification of the agarose, and electrophoresis was performed until the Bromophenol Blue indicator reached the bottom of the separation gel. The concentrations of separation gel and spacer gel were 15 and 7% respectively, and the current was 30 mA.

**Staining.** Coomassie Brilliant Blue R-250 was used for staining. The gel was solidified for 1 h in fixation fluid comprised of 50% anhydrous ethanol and 10% glacial acetic acid. The fixation fluid was removed and the gel was stained with 0.1% Coomassie Brilliant Blue R-250 and vibrated for 10 h. The

stain was removed by rinsing the gel twice with distilled water and adding destainer (30% anhydrous ethanol and 8% glacial acetic acid) and vibrating. The destainer was replaced until the background of the gel was clear.

**Silver staining and image scanning.** Following silver staining and coloration the ImageScanner (GE Healthcare Life Sciences) was used to obtain the 2-DE images. The protein spots were counted using ImageMaster 2D Platinum software, version 3.0 (GE Healthcare Life Sciences). Automatic identification of protein spots was conducted with the software, however, if the boundary between protein spots were clear, they were segmented into two spots.

**Gel image comparison.** The distribution of protein isoelectric points of the two types of protein extraction were compared according to the 2-DE gel image. BandsScan 5.0 (Glyko, Inc., Novato, CA, USA) was used as comparison software.

**In-gel digestion.** Samples were spotted onto a MALDI target plate with an equal volume of matrix solution, containing 5 mg/ml  $\alpha$ -cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid. An AutoFlex speed MALDI TOF/TOF MS (Bruker Corporation, Billerica, MA, USA) was used with a mass accuracy of 50 ppm following external calibration. The samples were analyzed in MS mode (for generation of peptide mass fingerprints) as well as in TOF/TOF mode (for fragmentation analysis of the highest intensity peaks). MS spectra were transformed into peak lists using the software flexAnalysis version 3.0 (Bruker Corporation). The peak lists of the MS and MS/MS spectra were merged using BioTools version 3.0 software (Bruker Corporation).

**Protein detection.** The amino acid sequence tags obtained from each peptide fragmentation in MS/MS analyses were used to calculate match scores and search for protein candidates in three groups using Mascot software, version 2.3.01 from Matrix Science (<http://www.matrixscience.com>). The retrieval parameters were as follows: Type of search, MS/MS Ion Search; enzyme, trypsin; fixed modification, Carbamidomethyl (C); variable modification: Gln->pyro-Glu (N-term Q), Oxidation (M); mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance,  $\pm 0.1$  Da; and fragment mass tolerance,  $\pm 0.1$  Da.

## Results

**2-DE imaging.** Samples from the two groups underwent 2-DE three times (500  $\mu$ g/sample) in the same environment, the images were scanned and the three images were identified to be comparable in each group (Figs. 1 and 2). The match scores were  $82.1 \pm 1\%$  in group A and  $83.2 \pm 2\%$  in group B.

**Differential analysis of protein spots.** A total of 10 different protein spots were identified by 2-DE of KOA synovial membrane samples in groups A and B; the homologous proteins, putative molecular weight and isoelectric point, and protein scores were determined (Fig. 3). Out of the 10 proteins, certain protein spots were identified to be the same, such as NO3, NO7 and NO8 (serum albumin). In samples of unilateral KOA

synovial membrane, NO1 was protein disulfide-isomerase and NO2 was creatine kinase (CK) M-type. NO6 was identified as lumican, NO10 was  $\alpha$ -2-HS-glycoprotein (AHSG) and NO4, NO5, and NO9 were designated as uncharacterized proteins from the bilateral KOA synovial membrane samples (Table I).

## Discussion

The synovial membrane is located within joint spaces and aids in the maintenance of normal joint function. Synovial membranes produce and secrete hyaluronan to lubricate the tissues of the joint, and serve an important role in the nutrition of cartilage, in addition to absorbing inflammation factors. Synovial fibrosis is a major contributor to joint stiffness in OA, which is elevated in OA and is key in the onset and persistence of synovial fibrosis. The process of synovial membrane lesions (from early inflammation to synovial hyperplasia), and the generation of inflammatory mediators and cytokines results in cartilage damage. Therefore, it is hypothesized that investigating and treating the cartilage alone in OA is not sufficient. Further investigation is required regarding the prevention of OA, to include the consideration of diverse pathogenic factors and taking an interdisciplinary approach, with the synovial membrane becoming a novel treatment target for OA, which may prevent joint structure damage and improve the clinical symptoms. In recent years, in order to further clarify the diagnostic biomarkers and prognostic indicators in different diseases, increasing numbers of studies are referring to the use of proteomics. Proteomes of degenerative/inflamed synovial membranes from rheumatoid arthritis (RA) and OA and a chronic arthritic condition, spondyloarthritis were previously investigated using 2-DE followed by tandem mass spectrometry (15).

To date, there are few studies regarding the proteomics of the synovial membrane. Furthermore, to the best of our knowledge, there are no studies reporting the differences between proteomics of the synovial membrane in spontaneous and secondary OA induced by a bilateral and unilateral ACLT model of KOA. Thus, the present study aimed to elucidate the differences in the proteomics of the synovial membrane using 2-DE in spontaneous and secondary KOA rabbit models, in order to establish the diverse remedies for OA resulting from the aging process and traumatic OA in the future. The results illustrated that the proteomics of the synovial membrane in the spontaneous and secondary KOA models were different. The proteins, disulfide-isomerase and CK M-type, were identified in the unilateral ACLT synovial membrane tissue, and serum albumin (three protein spots), lumican and AHSG were observed in the bilateral ACLT synovial membrane tissue. In addition, three protein spots were uncharacterized in the bilateral ACLT synovial membrane.

Protein disulfide-isomerases (PDIs) have been reported in different tumors and 19 family members have been identified. The function of PDI is to catalyze oxidative folding of novel peptide chains in the endoplasmic reticulum, in addition to participating in calcium homeostasis and antigen presentation. Procollagen and thyroglobulin, which are associated with PDI have been identified in previous studies (24). Li *et al* (25) investigated mechanical-stress loading-induced OA of the articulation mandibularis, and identified PDI in the mandibular

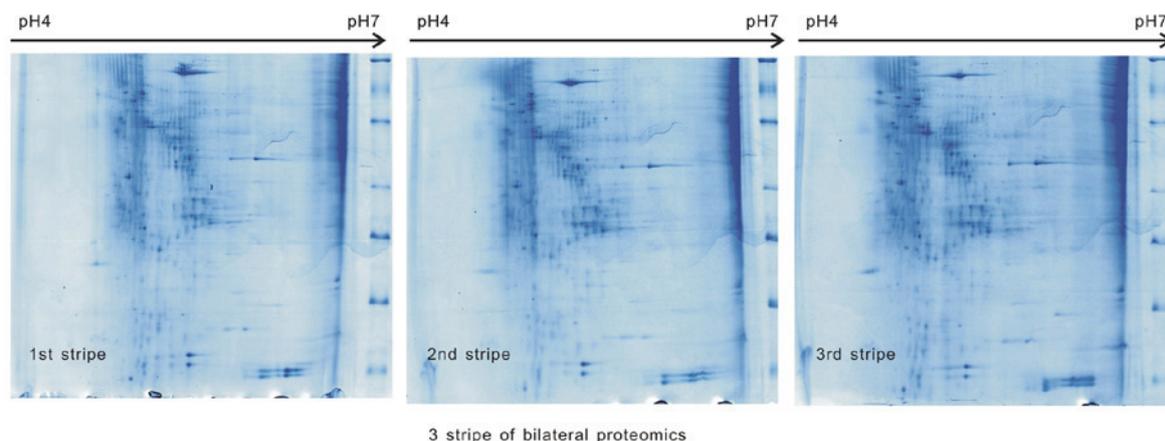


Figure 1. Images of three stripes from 2-DE of group A following bilateral anterior cruciate ligament transection. The image results for the three stripes from the same 2-DE are consistent, demonstrating that 2-DE of the synovium from group A was successful. 2-DE, two-dimensional polyacrylamide gel electrophoresis.

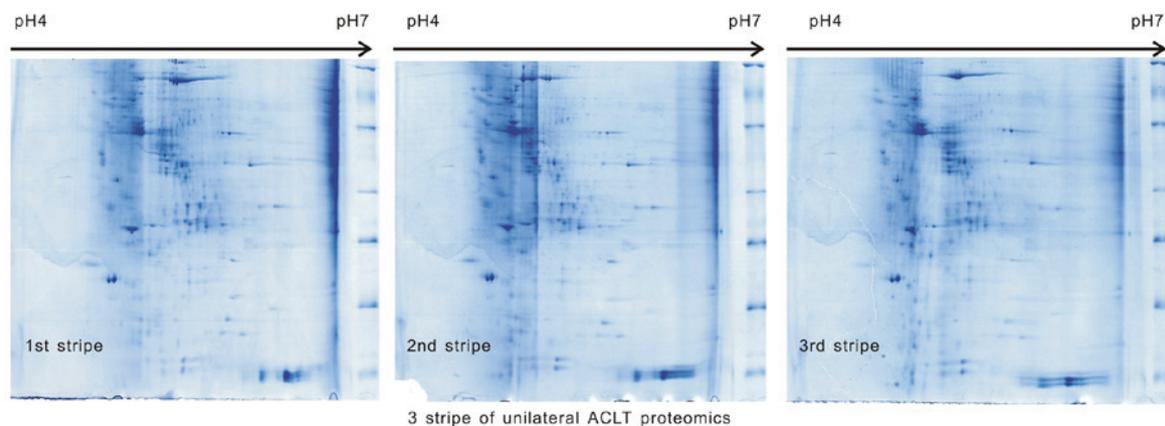


Figure 2. Images of three stripes from 2-DE of group B following unilateral anterior cruciate ligament transection. The image results for the three stripes from the same 2-DE are consistent, demonstrating that 2-DE of the synovium from group B was successful. 2-DE, two-dimensional polyacrylamide gel electrophoresis. ACLT, anterior cruciate ligament transection.

cartilage of rats. However, whether the synovial membrane of rabbits with KOA contains PDI has not, to the best of our knowledge, been documented thus far. In the current study, PDI was identified in the synovial membrane of unilateral ACLT, while it was not identified in bilateral ACLT. PDI affects protein metabolism, calcium homeostasis and procollagen synthesis, which all impact upon the pathological alterations in the tissues of the KOA joint. Thus, it was hypothesized that the PDI reduces collagen synthesis, which accelerates the process of KOA due to increased load in the bilateral ACLT joint.

In addition, the CK M-type protein was identified in the synovial membrane of unilateral ACLT, while it was not identified in bilateral ACLT. The components of synovial fluid, which are secreted by the synovial membrane, can be exchanged, and enter the circulatory system via synovial membrane capillaries, are potential biomarkers that can be detected in blood and urine (26). Therefore, the present study proposed that the varied expression levels of the CK M-type protein in the synovial membrane results from the difference in severity of the synovial membrane lesion between bilateral and unilateral KOA. As a result of the difference in CK expression levels between bilateral and unilateral KOA, the density of CK in the serum

is also varied; i.e. the CK density is downregulated in bilateral KOA. In addition, Chen *et al* (27) reported that the CK contents increase then reduce from onset to the later stages of RA. Eimre *et al* (28) proposed that OA was associated with increased sensitivity of mitochondrial respiration to ADP, causing a reduction in total activities of CK with marked reductions in the mitochondrial CK fraction. The authors suggested that due to degenerative remodeling occurring during the development of OA, these complexes become structurally and functionally impaired, resulting in increased access of exogenous ADP to mitochondria and dysfunction of the CK-phosphotransfer system. Borges *et al* (29) identified increased plasma activities of total CK (2.0-fold) in ballet dancers immediately after class, a finding that is significant in preventing the development of chronic conditions that are commonly observed in dancers, such as those with arthritis and synovitis.

Serum albumin is an essential material in cellular physical activity. Alterations in serum albumin content result in pathological alterations. Huang *et al* (30) demonstrated that the serum albumin status may be important in the utilization and metabolic turnover of plasma pyridoxal 5-phosphate in the presence of chronic inflammation and autoimmune disease, such

Table I. A total of 10 representative proteins of knee osteoarthritis rabbits from groups A and B identified by mass spectrometry of two-dimensional polyacrylamide electrophoresis gels of the synovium.

Spot no. (SSP)	Accession no. (in IPI_rabbit)	Homologous protein	Putative Mr (Da) / pI	Protein score
Group A				
NO1	P21195	Protein disulfide-isomerase	57172/0.29	207
NO2	P00563	Creatine kinase M-type	43313/0.27	1441
NO3	G1U9S2	Serum albumin	70916/1.04	546
NO4	G1ST52	Uncharacterized protein	182182/0.25	497
NO5	G1SWS9	Uncharacterized protein	53679/2.13	511
NO6	G1SP97	Lumican	38736/0.59	151
NO7	G1U9S2	Serum albumin	70916/4.96	1657
NO8	G1U9S2	Serum albumin	70916/1.50	545
Group B				
NO9	G1SP97	Uncharacterized protein	50151/3.51	728
NO10	G1SGQ5	$\alpha$ -2-HS-glycoprotein	39539/2.25	358

Mr, molecular weight; pI, isoelectric points; SSP, secondary structure of protein.

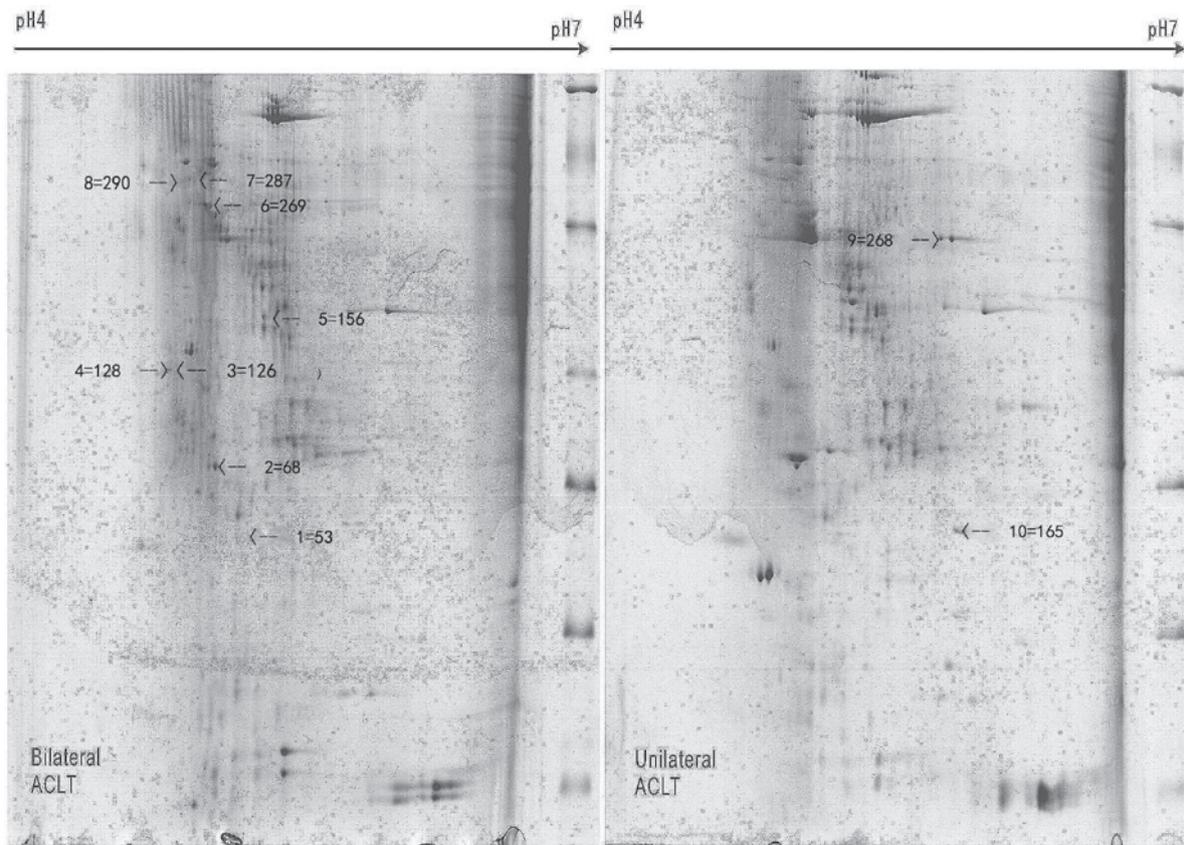


Figure 3. Protein spot distribution in synovial membranes from groups A (bilateral ACLT) and B (unilateral ACLT). ACLT, anterior cruciate ligament transection. Values are molecular weight divided by isoelectric point.

as in patients with RA. However, there are few investigations regarding the association between OA and serum albumin (31). In the present study, the serum albumin level in the synovial membrane of bilateral ACLT was observed (it was not identified in unilateral ACLT) with three of the protein spots identified as

serum albumin; thus, it was inferred that serum albumin levels may increase in the early-middle stage (6-week ACLT model) in order to increase the elimination of inflammatory factors. Therefore, regulating serum albumin levels may present as a novel method for treating OA.

Lumican is a leucine-rich proteoglycan and component of the extracellular matrix. Lumican and fibromodulin regulate the assembly of collagens into higher order fibrils in connective tissues. Jepsen *et al* (32) hypothesized that lumican and fibromodulin were candidate genes and key in the pathogenesis of certain types of Ehlers-Danlos syndrome and other connective tissue disorders. Previously, numerous lumican studies were regarding tumors, with few studies focusing on KOA (31-34). However, the association between lumican and OA or RA has been reported; Seki *et al* (33) identified that cultured RA fibroblastoid synoviocytes contain lumican protein, which encodes extracellular matrix components. Further investigation of lumican and fibromodulin may facilitate with the treatment of RA. In the present results, two protein spots were identified as lumican in the synovial membrane of bilateral ACLT, however not in unilateral ACLT (34). Fernández-Puente *et al* (34) identified serum protein biomarkers for moderate and severe OA, and identified six proteins that were only modulated in moderate OA, 13 proteins that were only modulated in severe OA and 7 that were modulated in the two; one of which was lumican. The authors indicated that the specificity and selectivity of these candidate proteins required validation prior to the development of novel molecular diagnostic or prognostic tests for OA. Melrose *et al* (35) observed that the fragmentation of small leucine-rich proteoglycans was increased in the degenerate osteoarthritic articular cartilage and menisci when compared with the articular cartilage of a normal knee. The authors suggested that specific decorin and fibromodulin core protein fragments in degenerate meniscus and/or human articular cartilage may be of value as biomarkers of disease, and further research may identify them as therapeutic targets. Clements *et al* (36) identified that the expression levels of lumican genes were increased in OA cartilage. Therefore, the present study proposed that lumican would be upregulated in OA, and that regulating lumican expression in the synovial membrane may present as a novel treatment method for OA, consistent with a previous study (35).

The role of AHSG in the augmentation of neutrophil phagocytosis by macrophages, thus acting as an anti-inflammatory molecule, was reported in 1961 (37). Heiss *et al* (38) suggested that AHSG is a systemic inhibitor of precipitation of basic calcium phosphate, preventing unwanted calcification, and that AHSG domain D1 is most efficient in inhibiting basic calcium phosphate precipitation (38). Liu *et al* (39) demonstrated that the AHSG gene may contribute to bone size variation at the hip in a Chinese population. In addition, Nishio *et al* (40) observed that AHSG exerted mild inhibitory effects on calcium oxalate crystallization, and that low urinary concentrations of prothrombin F1 and osteopontin may contribute to stone formation. According to further findings, numerous studies have identified that AHSG is a non-specific opsonin, with its serum level being demonstrated to vary in patients who have experienced trauma, or who have diabetes mellitus. Lebreton *et al* (41) identified that the serum level of AHSG was negatively correlated with the acute phase reactants. A previous study by Mbuyi-Muamba *et al* (42) reported that treatment of RA did not appear to modify AHSG plasma levels; thus, the probable biological role of AHSG in RA is debated. However, Saroha *et al* (43) reported that the level of plasma AHSG was reduced by two-fold in RA patients when

compared with healthy control subjects. To date, the association between OA and AHSG has required further elucidation. In the present study, the synovial membrane sample contained AHSG in the bilateral ACLT group while it was not observed in the unilateral ACLT group. The present study proposes that during the onset of OA, the synovial membrane expression of AHSG may be reduced for anti-inflammatory purposes or to induce the hyperostosis.

The proteomics in the synovial membrane of spontaneous and secondary KOA models were compared in the present study. As all 10 proteins have not been reported in the synovial membrane, their functions in KOA can only be hypothesized. The present study identified PDI and CK M-type in the unilateral KOA model, but not in the bilateral KOA model (and more severe pathological changes than unilateral KOA). PDI may accelerate the process of KOA, as it regulates protein metabolism, calcium homeostasis and procollagen synthesis. The levels of CK may reflect the different disease phases or the degree of pathology of KOA; with upregulated CK concentrations in the early phase/light KOA and downregulated CK concentrations at the mid to late phase/severe KOA.

Serum albumin, lumican, AHSG and three uncharacterized proteins were observed in the bilateral KOA model, but not in the unilateral KOA model. It is hypothesized that the serum albumin levels increase to inhibit the KOA process. The lumican levels in the synovial membrane may induce cell proliferation in connective tissues resulting in synovial hyperplasia, and AHSG proteins in the synovial membrane may be secreted into the synovial fluid, stimulating bone formation and resulting in hyperostosis.

In conclusion, the present results demonstrate the differential proteomic expression and indicate the diverse pathomechanisms between bilateral and unilateral KOA, highlighting that spontaneous and secondary KOA require diverse methods of treatment. Regulation of PDI and CK M-type expression levels may be necessary in secondary KOA or in the early phase of spontaneous KOA, and reduced lumican and AHSG levels in spontaneous KOA, particularly at the mid to late phase, as the majority of patients are at this stage upon OA diagnosis. However, further investigations regarding the different mechanisms of synovial membrane proteomics in spontaneous and secondary KOA are required.

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