

## Original Article

# Correlation of synovial tissue protein abundance with menopause in osteoarthritis

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Received April 16, 2022; Accepted September 7, 2022; Epub October 15, 2022; Published October 30, 2022

**Abstract:** Background: Osteoarthritis (OA) is a common articular disorder. Epidemiologic surveys show a higher prevalence of OA in women than men and that morbidity is higher during menopause. We aimed to explore whether menopause influences the clinical recovery of a knee joint following OA and injury, and identify associated mechanisms by analyzing the proteomic profile of synovial tissue (ST) samples. Methods: Routine blood examination and hormone level tests were conducted before surgery. ST samples from eight participants were collected intraoperatively for proteomic analysis. One day before and one month after the surgery, we assessed various aspects of function in the affected knee including the with Visual Analog Score (VAS), Lysholm, The Western Ontario, and McMaster Universities Osteoarthritis Index (WOMAC) scores. The relationships between proteomic data, estrogen levels, and affected knee function were compared and analyzed. This was a retrospective study. Result: Menopause was associated with the clinical outcomes of knee OA and knee injuries. ST proteomic data identified that 80 proteins in premenopausal OA females were significantly different from menopausal OA females. In addition, 100 proteins were significantly different between premenopausal OA females and premenopausal injured females. Conclusions: Age and menopause showed a positive correlation with the protein profile of ST from OA or knee injury female patients, indicating that the protein components might be affected by menopause. Postoperative clinical outcomes were affected by menopause. We conclude that menopause may, in part, regulate knee joint function by altering ST protein expression.

**Keywords:** Proteome, osteoarthritis, synovial tissues, menopause female

## Introduction

Osteoarthritis (OA) is a common joint disorder manifesting joint pain and limited activity and function, which seriously affects patient quality of life. OA pathogenesis remains incompletely understood. Currently, diagnosis relies mainly on aberrations in cartilage morphology as determined by magnetic resonance imaging, ultrasound, and arthroscopy, lag, traumatic, such as limit when the diagnosis of OA patients tend to have different degree of damage of articular cartilage, so looking for effective early diagnosis of OA index for early intervention to delay the disease, Prevention of irreversible degeneration of the affected joint critical. Previous studies have reported risk factors for the initiation and development of OA, including

age, estrogen, sex, dietary intake, and other variables [1-5]. Menopause has also been proposed to be associated with the onset and progression of OA in females [6].

OA is not only a degenerative disease of cartilage or subchondral bone, but also affects the synovial tissues (ST) at various stages of OA. ST is critical to normal joint function and its impairment underlies many common diseases [7]. Essential components expressed in ST help regulate knee joint function and joint responses to various disorders. For example, the prolactin receptor (PRLR), expressed by macrophages, can be detected in ST samples obtained from individuals with OA and rheumatoid arthritis (RA). It activates macrophages and may stimulate the release of pro-inflammatory cytokin-

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es [8-10].  $\beta$ 1,4-galactosyltransferase-I ( $\beta$ 1,4-GalT-I) has been reported to be elevated in ST of OA patients [11]. However, other ST proteins that may also contribute to development of OA remain unidentified. Therefore, we aimed to explore the relationship between menopause and OA by studying the expression of ST protein in menopausal females with OA. This is a retrospective study.

### Methods

#### *Participants*

ST samples were obtained from female patients with a knee injury who had undergone arthroscopic synovectomy and meniscal repair, or female patients with knee OA who had undergone total knee arthroplasty and synovectomy from November of 2018 to April 2019 in Sichuan Provincial People's Hospital, China. The inclusion criteria included: (1) female sex, (2) OA group met the diagnostic criteria of OA revised by the American Rheumatic Association in 1995, (3) knee injury group presented with simple meniscus and/or knee cruciate ligament injuries, (4) aged 20-70, (5) no associated bone and joint diseases, such as rheumatoid arthritis, gout arthritis, knee fracture, etc., (6) no previous knee surgery, (7) no intraarticular injection of non-steroidal drugs or immunomodulatory drugs within 3 months of surgery, (8) provided informed consent and was willing to be followed up.

#### *Detection of blood plasma hormone levels*

Venous blood used to quantify estrogen was collected on the morning of the operative day by nursing staff and was immediately sent to the Hospital's test center.

#### *Protein preparation and digestion*

ST samples were harvested during surgery and immediately stored in liquid nitrogen. Protein extraction was conducted according to the requirements of mass spectrometry (MS) analysis. Briefly, 20 mg of sample was ground and sonicated (Q800R sonicator, QSONICA, Newton, Connecticut, USA) in 1 ml lysis buffer (7 M urea, 4% SDS, 1 mM phenylmethane sulfonyl fluoride (PMSF), 2 mM EDTA, 10 mM dithiothreitol (DTT), 1 $\times$  protease inhibitor cocktail (Sigma-Aldrich, St. Louis, USA) in 30 mM HEPES, pH 7.4). The insoluble content in the

lysate was discarded by centrifugation at 13000 g and 4°C for 10 min. The total protein concentration of the supernatant was tested with a bicinchoninic acid assay (BCA) Protein Kit (Thermo Fisher Scientific, Bonn, Germany) and the quality of the samples was evaluated via the SDS-PAGE method. The samples were then precipitated with 15% trichloroacetic acid for 2 h at 4°C and washed with acetone. The precipitates were redissolved in triethylammonium bicarbonate buffer. The solutions were then digested with trypsin (Promega, Madison, WI, USA) at an enzyme/substrate ratio of 1:50 at 37°C for 16 h.

#### *TMT labeling, HPLC fractionation*

The digested peptide mixtures were first desalted using a Sep-Pak C18 column (100 mg, 1 cc; Waters Corporation, Milford, MA, USA). TMT labeling was conducted according to the manufacturer's instructions. Briefly, anhydrous acetonitrile was mixed with TMT Reagents pre-equilibrated to room temperature. The mixture was added to each sample, and the purified samples were chemically labeled with the TMT10plex™ Isobaric Label Reagent Set (Thermo Scientific, Rockford, CA, USA) for 2 h at RT. The samples were then pooled and dried by vacuum centrifugation. The dried samples were acidified with 0.1% trifluoroacetic acid and desalted with Sep-Pak C18 (100 mg, 1 cc; Waters Corporation, Milford, MA, USA) and fractionated through a BEH C18 column (2.1 $\times$ 150 mm, 1.7  $\mu$ m, 130 Å) using ACQUITY UPLC® H-Class System (Waters Corporation, Eschborn, Germany) at a flow rate of 250  $\mu$ l/min.

#### *LC-MS/MS analysis*

The eluted fractions were collected and combined into 12 fractions for further LC-MS/MS analysis. The settings of MS were as follows: ESI voltage, 2 kV; inlet capillary temperature, 300°C; full-scan automatic gain control (AGC) target, 5 $\times$ 10<sup>5</sup> ions at 60,000 resolution; scan range, 400-1600 m/z; Orbitrap full-scan maximum injection time, 50 ms; MS/MS scan AGC target, 2 $\times$ 10<sup>5</sup> ions at 30,000 resolution; and maximum rejection, 150 ms.

#### *Evaluation of clinical outcomes*

One day before and one month after surgery, joint function was evaluated using a Pain Visual Analog Score (VAS), Lysholm, The Western

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**Table 1.** Patients characteristics

Variable	Postmenopausal OA female	Premenopausal OA female	Premenopausal knee injury female
Number (n)	3	3	2
Mean age (M ± SD, y)	63.3±5.8	34.7±5.7	40.5±0.7
Affected knee (left/right, n)	2/1	1/2	2/0
BMI	24.7±1.2	21.3±1.1	20.6±0.3
Estrogen (pg/mL)	< 11.8*	138.9±48.4	101.1±35.9
Evaluated scores (pre/one-month VS post-surgery)			
VAS	6.7±0.58 VS 2.0±1.0	6.3±0.6 VS 1.3±0.6	6.5±0.7 VS 1.0±0.1
Lysholm	69.3±1.16 VS 81.3±4.0	65.3±5.0 VS 92.3±2.1	67.0±1.4 VS 90.5±2.1
WOMAC	148.7±7.02 VS 106.3±10.0	116.7±20.8 VS 71.0±3.6	144.0±5.7 VS 69.0±1.4

BMI: body mass index (normal BMI ranged from 18.5 to 24); VAS: the visual analog scale (VAS score: 0 represents no pain; Less than 3 points indicates slight pain; 4-6 points, the pain is more obvious; 7-10 points, the pain is very severe and unbearable). Lysholm: Lysholm knee score (The lower the Lysholm index, the higher the OA. A score above 95 is excellent, 94 to 85 is good, 84 to 65 is fair and less than 65 is poor); WOMAC: the Western Ontario and McMaster Universities Osteoarthritis Index. (The higher the WOMAC index, the more severe OA, mild < 80, moderate 80-120, severe > 120); \*Estrogen levels of post-menopausal women are all less than 11.8.

Ontario and McMaster Universities Osteoarthritis Index (WOMAC) [12]. All tests were conducted by the same group of doctors.

### Protein reactions and pathways

Proteins linked to the immune or endocrine systems that showed low insignificant abundances between old OA patients and young OA patients were selected out using the ClueGO tool in Cytoscape (v3.7.2) and were analyzed with the online Reactome database (<https://reactome.org/>).

### Statistical analysis

The raw proteomic data were initially processed with Proteome Discovery 1.4 (Thermo Fisher Scientific, Waltham, MA, USA). KOBAS 3.0 (<http://kobas.cbi.pku.edu.cn/>) [13], a web server for gene/protein functional annotation and functional gene set enrichment, was used for pathway and Gene Ontology functional category enrichment analysis. False discovery rates (FDR) were calculated according to the method of Benjamin. Significance results was determined based on an FDR-adjusted *p*-value ≤ 0.05. IBM SPSS version 17.0 (IBM Corp, Armonk, NY) was used for the data analysis. *P* value < 0.05 was considered significant. Summary data of BMI, age, VAS, Lysholm, and WOMAC scores are shown as the mean ± standard deviation. Student's *t*-test and one-way ANOVA were used to compare group differences, as appropriate. Pearson's correlation and

linear regression analysis were used to evaluate the relationship between the concentration of estrogen and the VAS, Lysholm and WOMAC scores.

### Results

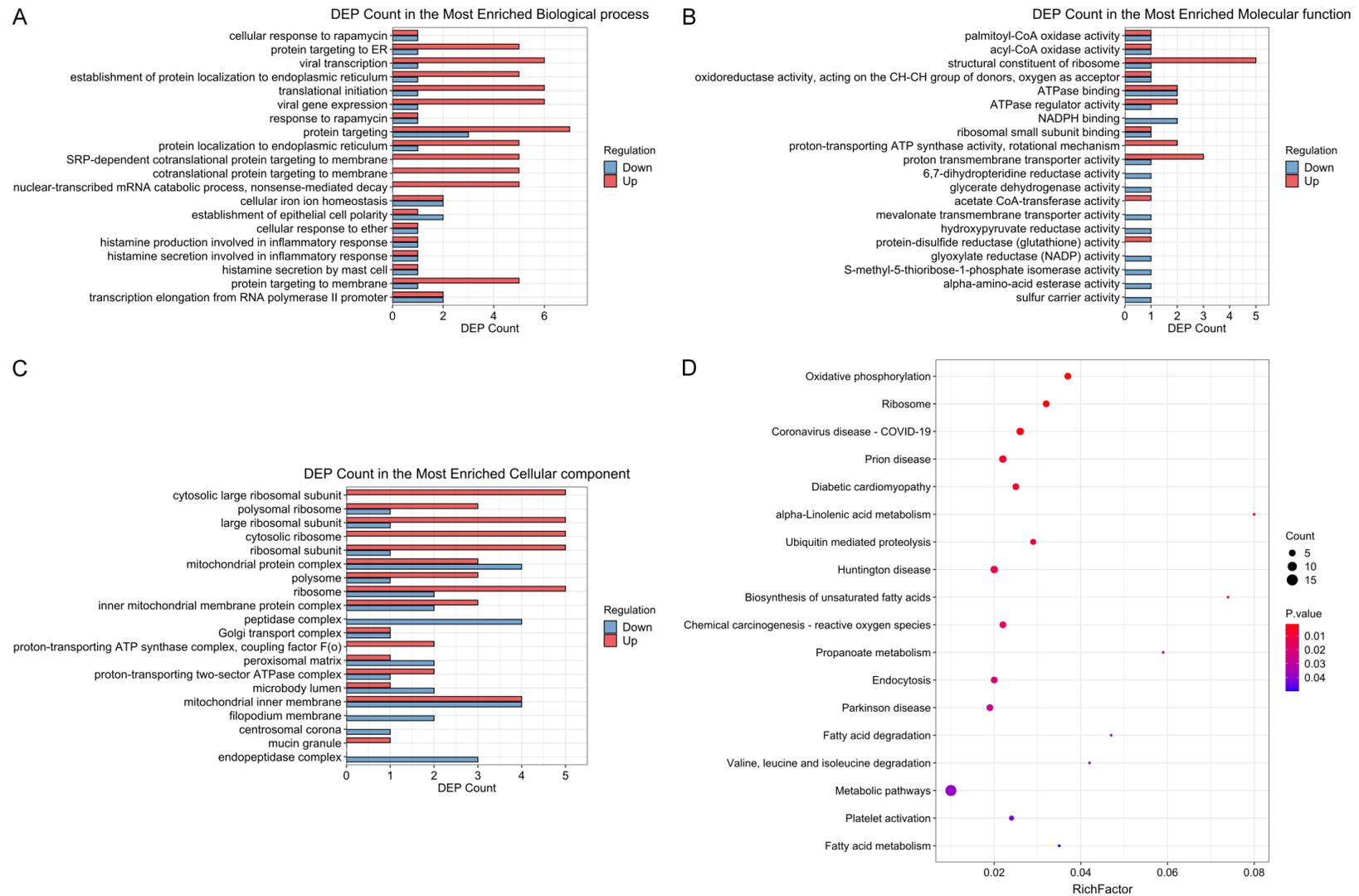
#### *The effects of menopause on the clinical outcomes of knee OA and injury*

8 individuals met our inclusion criteria and were included in the analyses. The basic participant characteristics, the estrogen levels, and pre, and post-surgery scores are summarized in **Table 1**. Menopause and estrogen levels were associated with post-surgery functional outcomes. Although all participants improved one month after surgery, premenopausal females showed greater functional improvement than menopausal women (*P* < 0.05). However, we found no differences in post-surgical functional recovery between women with high versus low estrogen.

#### *Characteristics of the premenopausal OA and knee injury ST proteome profile*

We detected approximately 4000 proteins in the samples; of these, 3465 proteins were used for the analysis (*P* < 0.01). The proteins were composed of different cellular components and were involved in various biologic processes (**Figure 1**), according to GO and KEGG analysis.

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**Figure 1.** GO and KEGG analysis of synovial tissues (ST) from premenopausal OA women and premenopausal knee injury women. A. GO analysis of the biological process of proteins in ST. B. GO analysis of the molecular function of proteins in ST. C. GO analysis of the cellular component of proteins in ST. D. The biologic processes of the detected proteins analyzed by KEGG.

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**Table 2.** The top 20 proteins according to variation in different groups

post- vs premenopausal				OA vs knee injury		
Accession	Accession	GeneSymbol	Log2 (post/pre)	Accession	GeneSymbol	Log2 (OA/injury)
O43488	Q9BTE1	DCTN5	0.289498664	Q9NXG2	THUMPD1	0.269383267
Q9GZT8	O43488	AKR7A2	0.409239429	Q96T23	RSF1	0.277527593
P30876	Q9NX24	NHP2	0.302979617	Q9ULT0	TTC7A	0.289193504
P07333	O00160	MYO1F	0.275863833	Q9Y5P4	COL4A3BP	0.290021778
Q9Y2S2	P52630	STAT2	0.294097071	P63172	DYNLT1	0.296420131
Q658P3	Q15257	PTPA	0.283669719	Q9UKK9	NUDT5	0.316220545
Q5SRE7	P07333	CSF1R	0.443399472	O95858	TSPAN15	0.348805567
P48426	O14773	TPP1	0.31591994	Q9NX14	NDUFB11	0.395980419
Q15006	Q15006	EMC2	0.682535623	Q13523	PRPF4B	0.521081568
Q9BRG1	Q9Y2S2	CRYL1	0.399596671	P07947	YES1	0.6417041
Q00013	P55039	DRG2	0.373345031	P41219	PRPH	0.906690157
Q96G23	Q9Y3E1	HDGFL3	-0.298723752	Q9Y570	PPME1	-0.502189867
Q01844	O75592	MYCBP2	-0.281459426	O95219	SNX4	-0.440872985
O00487	P62841	RPS15	-0.371475399	Q15370	ELOB	-0.360115548
O95182	Q9UMS6	SYNPO2	-0.441555468	Q13232	NME3	-0.342458488
P53992	Q2TAA2	IAH1	-0.309676687	Q15427	SF3B4	-0.274833517
Q9UK45	Q99729	HNRNPAB	-0.325274373	Q13445	TMED1	-0.273530095
Q9NZD2	Q13620	CUL4B	-0.381895597	P56545	CTBP2	-0.47964211
P05388	P14621	ACYP2	-0.329073087	Q14938	NFIX	-0.435821005
P61513	O14495	PLPP3	-0.385721448	Q15628	TRADD	-0.362026664

The average counts of the top 20 candidate proteins that are most affected by menopause or disease types estrogen levels.

### Premenopausal OA and knee injury ST protein profile in ST

We found that ST protein expression patterns were affected by menopause, and disease types (OA vs. injury). The average counts of the top 20 candidate proteins which would be most affected by menopause or disease types' estrogen levels are listed in **Table 2**.

To better understand whether the protein profiles were affected by estrogen, we quantified blood estrogen levels. The differentially expressed proteins are shown in **Table 2**. The relationship between estrogen levels and protein profiles was tested using Pearson correlation analysis. There was no significant correlation between protein expression patterns and estrogen levels.

ST protein abundance differed between menopausal OA and postmenopausal individuals (**Figure 2A** and **2C**). Comparing postmenopausal OA and premenopausal OA females, LSM7, FOLR2, and PRL37A were significantly increased, while ABAT, STEAP3, and PPM18 were markedly decreased in the 1.2 enrichment

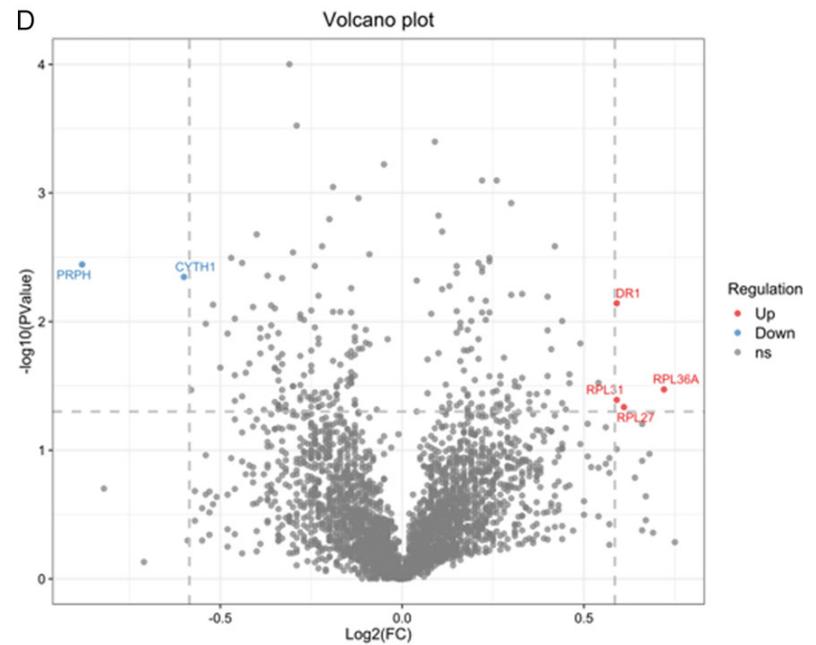
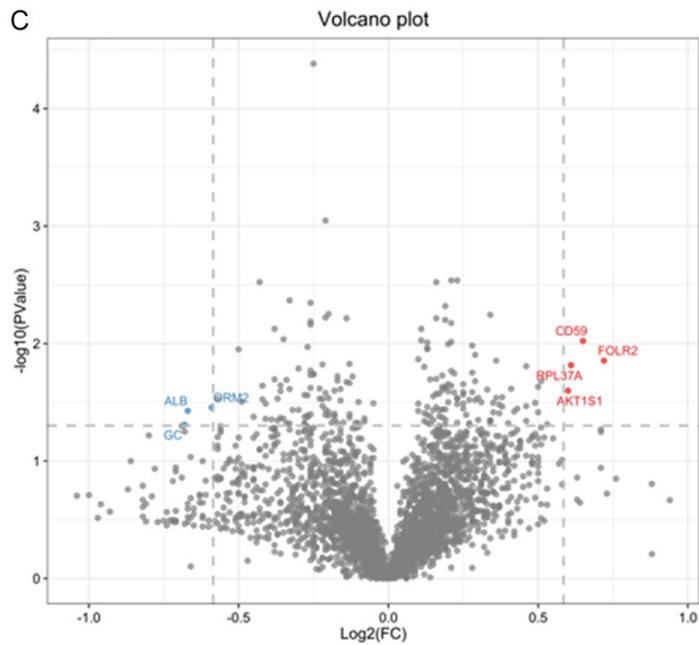
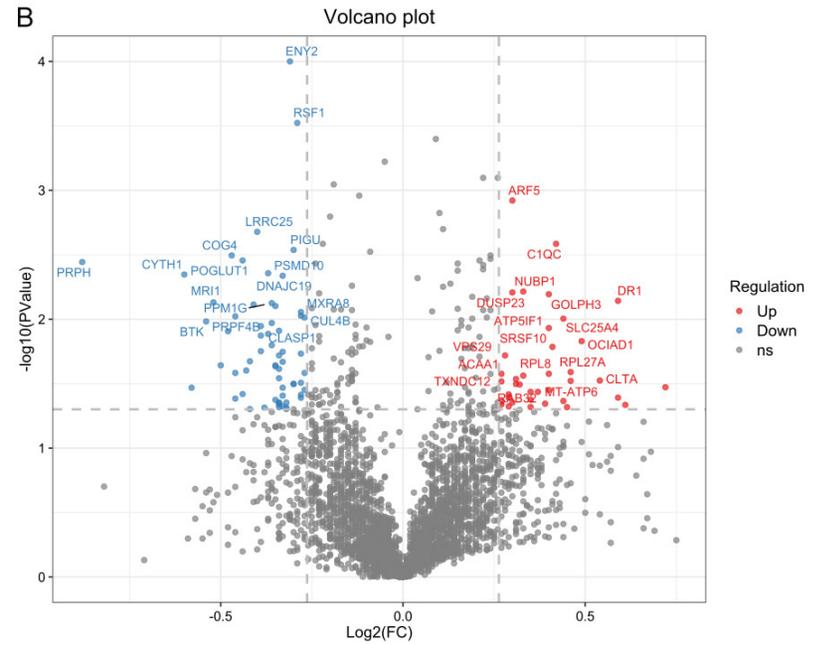
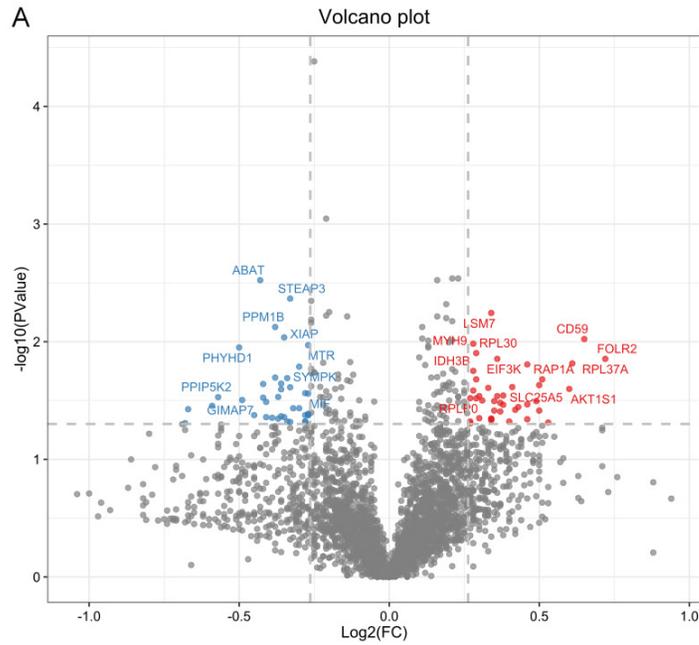
times (**Figure 2A**). CD59, FOLR2, AKT1s1, and BPL37A were significantly increased, while proteins like ALB, GC, and ORM2 were markedly decreased in the 1.5 enrichment times (**Figure 2C**).

Interestingly, the ST protein profiles of premenopausal knee injuries differed from premenopausal OA individuals (**Figure 2B**). Compared to premenopausal knee injuries, premenopausal OA exhibited higher I ARF5, C1QC, and DR1 but lower ENY2, RSF1, and PRPH in the 1.2 enrichment times. In the 1.5 enrichment times, premenopausal OA female showed higher DR1, PRL31, and PRL36A; but lower PRPH, and CYTH1.

### Network analysis of candidate proteins

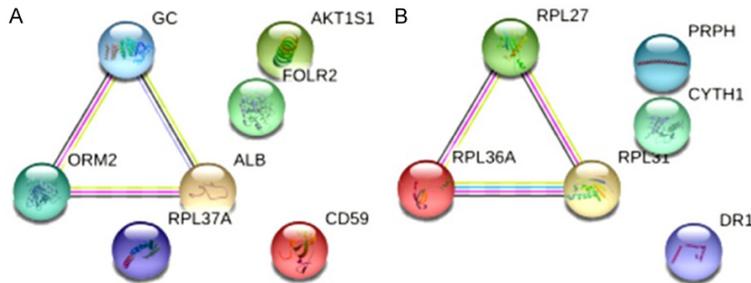
Candidate proteins affected by menopause, disease types and estrogen levels were further analyzed by network analysis. The protein-protein interaction network shown in **Figure 3**. (**Figure 3A** shows candidate molecules significantly influenced by menopause and **Figure 3B** shows those correlated with disease types).

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**Figure 2.** Differently expressed proteins in synovial tissues (ST) samples from different groups. A. Under the enrichment factor of 1.2, volcano plots of the differently expressed proteins between postmenopausal OA women and premenopausal OA women. B. Under the enrichment factor of 1.2, volcano plots of the differently expressed proteins between premenopausal OA women and premenopausal knee injury women. C. Under the enrichment factor of 1.5, volcano plots of the differently expressed proteins between postmenopausal OA women and premenopausal OA women. D. Under the enrichment factor of 1.5, volcano plots of the differently expressed proteins between premenopausal OA women and premenopausal knee injury women.



**Figure 3.** The protein-protein interaction network analyzed by STRING. A. Protein-protein interaction network of the proteins varied greatly with menopause in synovial tissues (ST) of knee osteoarthritis (OA) women. B. Protein-protein interaction network of the proteins statistical different between premenopausal OA women and premenopausal knee injury women.

### *The reactions and pathways analysis of candidate proteins analyzed reactome*

The proteomic data were further analyzed in the Reactome. Because the development of OA is associated with the immune and endocrine systems, candidate proteins whose abundance differed between menopausal and premenopausal participants were extracted and those related to the immune or endocrine system were then selected. These selected proteins were analyzed by “Reactions and Pathways” in Reactome. The potential reactions and pathways among these proteins are shown in **Figure 4**. This may provide insight into the mechanism through which menopause contributes to the development of OA.

### **Discussion**

Osteoarthritis (OA) is a leading cause of joint pain and disability, affecting a substantial proportion of people worldwide [14]. Significant efforts are being made to identify the synovial fluid protein components and their potential roles in OA [15-19]. Animal models of OA have been used to clarify understand the pathophysiology of OA and develop successful treatment of OA [20, 21]. However, the role of knee ST in the onset and development of OA remains poorly understood. In this study, we explored

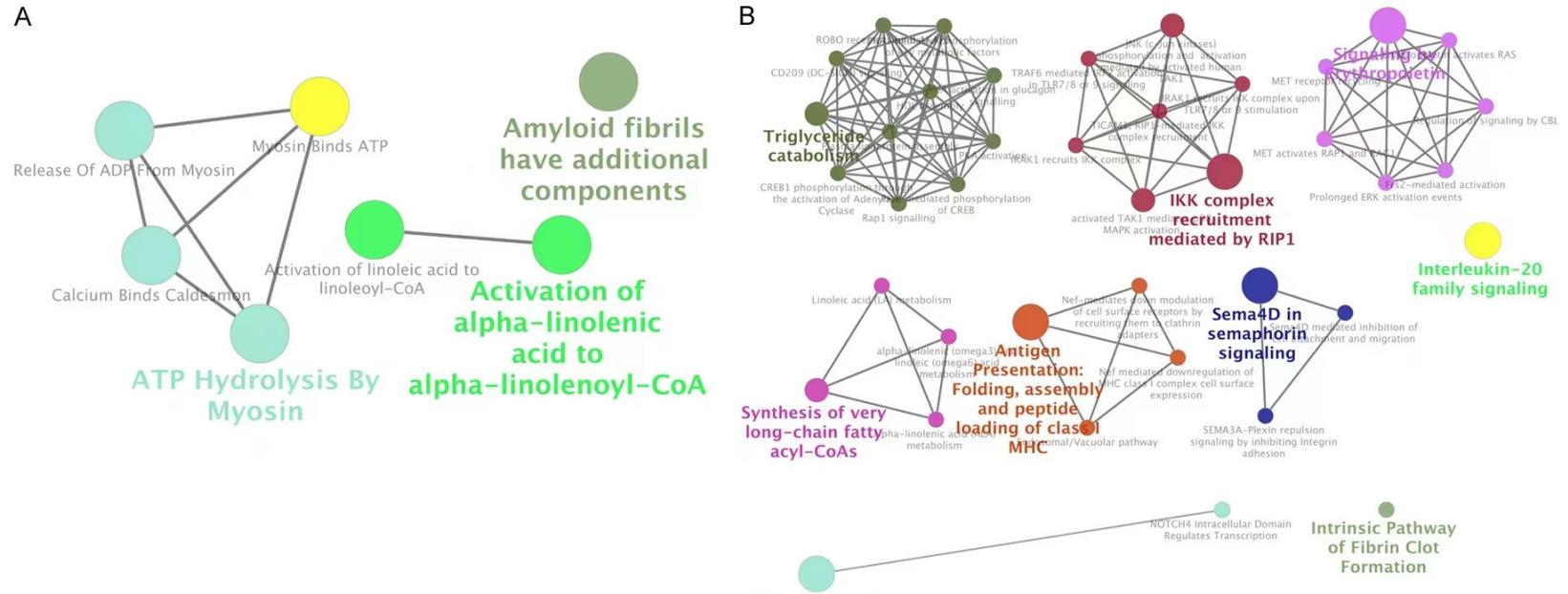
the relationship between post-operative knee joint improvement, menopause, and ST proteins in OA individuals. Our primary aim was to provide insight into mechanisms of OA onset and development.

As shown, menopause was associated with post-operative recovery knee OA and injury patients. However, we found no such relationship with estrogen levels. One possible reason is that estrogen levels may fluctuate and a single test

might not adequately capture clinically meaningful differences. The role of estrogen in OA development inconclusive. Some have reported that estrogen contributed to the recovery from OA and may reduce the prevalence of OA [22]. In vivo OA animal models also suggest that estrogen possesses anti-inflammatory characteristics [23]. By inhibiting the development of joint swelling and reducing the release of inflammatory cytokines, estrogen may effectively relieve joint pain and improve joint swelling. On this basis, estrogen treatments have been tried in OA. Unfortunately, the results of these trials are inconsistent [22, 24, 25].

We demonstrated that the ST protein profiles varied with menopause and disease type (OA vs injury). The protein composition of postmenopausal OA differed from that of premenopausal OA individuals. Our findings are consistent with previous reports [5]. However, the exact link between OA and menopause remains unclear. While we demonstrate marked differences associated with menopause, the specific causal links and mechanisms will need to be investigated. Menopause is associated with altered gene expression. However, to our best knowledge, it is not known whether estrogen levels have similar effects in the ST. We found that in our cohort, estrogen levels were not associated with differences in ST protein expression this

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**Figure 4.** Reactions and pathways analyzed by Reactome. The reactions (A) and the pathways (B) among the immune or endocrine-related proteins varied significantly in synovial tissues (ST) between menopausal and premenopausal osteoarthritis (OA) women.

stands in contrast to the relationship between menopause and protein in our cohort. This paradox might be the result of the dynamic fluctuation of estrogen levels in premenopausal women that we could not capture in the present study.

It had been shown that, compared to normal or ACL injured individuals, mRNA and protein levels are altered in synovial fluid, synovium, and peripheral blood from OA patients [26]. These changes are thought to contribute to the initiation of OA and may be biomarkers of joint disease.

We also found proteins that were differentially expressed among the different groups (**Figure 3**). Although we identified proteins that might be altered by menopause and OA, the pathways through which they contribute to joint dysfunction remain unknown. Protein-protein interactions might provide important clues for future studies to investigate the molecular mechanisms involved.

The reactions and pathways among immune or endocrine-related proteins that varied significantly in menopausal and premenopausal women were analyzed and visualized by the Reactome program (**Figure 4**), which might provide insight into the molecular mechanism of aging contributing to the development of OA. The deficiency of this study is the small sample size.

### Conclusions

We present the knee synovial tissue protein expression profiles from female OA and injury patients. We identified around 4000 proteins, of which 3465 were included in our analyses. We found that ST protein expression was associated with menopause. Similarly, menopause also influenced the patient post-surgery functional outcomes. Therefore, menopause might affect the development of OA as well as the post-surgery recovery of the knee joint by changing the protein expression patterns in the f ST. These ST proteins may offer biomarkers and therapeutic targets for OA. The exact effect of these proteins on the occurrence, development, and rehabilitation of OA remain unclear. More studies are needed to elucidate how aging, menopause, and the ST affect the knee joint.

### Acknowledgements

The authors would like to thank the participants for their contribution and cooperation and Jiguang Gene Biotechnology Co. Ltd (Nanjing) for their help in proteome analysis. This work was funded by Science & Technology Department of Sichuan Province (NO. 2019YFS0318); Foundation of Tianjin Health Commission (ZC20192); These fundings are used for data collection, hormone levels detection, proteome analysis and interpretation of data.

### Disclosure of conflict of interest

None.

### Abbreviations

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; OA, Osteoarthritis; ST, Synovial Tissue; WOMAC, The Western Ontario and McMaster Universities Osteoarthritis Index; VAS, Visual Analogue Score.

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