

Correlation of Synovial Tissues Protein Abundance With Age and Menopause in Osteoarthritis and Knee Joint Injury of Female Patients

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Research Article

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Abstract

Background

Knee injury and osteoarthritis (OA) are common articular disorders that affect numerous people of different ages, particularly menopausal women. Epidemiological surveys conclude that ladies gain a higher prevalence of OA than men, the menopausal women even get higher morbidity. However, studies about OA animal models suggest that the animals could remain a good joint function regardless of aging and menopause; age and menopause seem to have little effect on joint function. Thus, the study aimed to explore whether age and menopause will influence the clinical recovery of a knee joint in OA and injury women and the relevant mechanism by analyzing the proteome profile of synovial tissue (ST) samples.

Methods

The routine blood test and hormone levels were detected before the surgery. The ST samples of participants were collected during the surgery and 8 ST samples were used for proteome analysis. Just the day before and one month after the surgery, scores of Visual Analogue Score (VAS), Lysholm, The Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) were used for assessing the functions of the affected knee. The relationship among proteome data, aging, estrogen level, and affected knee function were analyzed.

Results

Age, menopause and disorders of the knee were related to the clinical outcomes of knee OA and injuries of female patients. Proteome data revealed that the abundance of 174 proteins in old women was statistically different from those of young women. Similarly, compared to the menopausal women, about 120 proteins were significantly changed in the samples taken from premenopausal women. 58 proteins were shown to be insignificant different amounts in ST from knee OA women compared to knee injury ones. The reactions and pathways of these proteins were analyzed and visualized.

Conclusions

Age and menopause showed a certain correlation with the protein profile of ST from OA or knee injury female patients, which indicated that the protein components might be affected by age and menopause. Besides, age and menopause also influenced the clinical outcomes of these patients after surgery therapy. Aging and menopause might take part in the regulation of remaining knee joint functions via modeling the ST protein expression.

Background

Osteoarthritis (OA) is a common complaint in young and old people, causing the patients many inconveniences in daily life and work. Therefore, epidemiological studies and controlled trials are carried out by many researchers. Based on the previous surveys, age, estrogen, sex, dietary intake et al are important risk factors for the initiation and development of OA [1,2,3,4,5]. Besides, menopause is also suggested to be associated with the onset and progression of OA in women [6]. The involvement of all of the above risk factors in the progression of osteoarthritis requires further research.

Synovial tissues (ST) have also been shown to be involved in remaining the joint function and the incidences of many joint diseases[7]. Essential components expressed in ST are of great significance in regulating the functions of the knee joint and the responses to multifarious joint disorders. For example, the prolactin receptor (PRLR), expressed by macrophages, is detected in ST samples from both OA and rheumatoid arthritis (RA) patients, stimulating the activation of macrophages with other pro-inflammatory cytokines [8,9,10]. β 1,4-galactosyltransferase-I (β 1,4-GalT-I) was also reported to be elevated in ST of OA patients [11]. However, many other proteins that might also contribute to the incidence and the development of OA in ST are not fully identified. Therefore, in the study, we purpose to explore the association of aging, menopause and OA by characterized ST proteomic expression patterns together with clinical outcomes.

Methods

Participants

Synovial tissue samples were obtained from female patients who suffered from knee OA or knee injury and underwent surgical therapy from Nov. of 2018 to April of 2019 in Sichuan Provincial People's Hospital. To be specific, patients who fitted the following criterion were included: (1) female patients with knee OA or knee injury, (2) aged at 20~70y, (3) without other maladies of bone and articular, or any pre-therapy history of the knee OA or knee injury, (4) signed the study consent and was willing to be follow-up. Although 21 patients were included, only samples from 8 patients were applied for proteomic analysis regarding their comparability.

Detection of blood plasma hormone levels

The peripheral blood used for estrogen testing was obtained on the morning of the operative day by nurses and was sent to the Hospital's testing center immediately.

Protein preparation and digestion

The synovial tissue (ST) samples were obtained during the surgery and immediately stored in liquid nitrogen. Protein extraction was performed according to the requirements of MS analysis. Briefly, 20mg of ST sample was ground and sonicated (Q800R sonicator, QSONICA, Newton, Connecticut, USA) in 1ml lysis buffer (7M urea, 4% SDS, 1mM phenylmethane sulfonyl fluoride (PMSF), 2mM EDTA, 10mM dithiothreitol (DTT), 1×protease inhibitor cocktail (Sigma-Aldrich, St. Louis, USA) in 30mM HEPES, pH 7.4). The insoluble content in the lysate was discarded by centrifugation at 13000g and 4 °C for 10min. The protein concentration of the supernatant was determined with a BCA Protein Assay 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 16h.

TMT labeling, HPLC fractionation

The digested peptide mixtures were first desalted using a Sep-Pak C18 column (100mg, 1cm³; Waters Corporation, Milford, MA, USA). TMT labeling was performed 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 2h at RT. The samples were then pooled and dried by vacuum centrifugation. The dried samples were acidified with 0.1% trifluoroacetic acid and desalted using Sep-Pak C18 (100mg, 1cm³; Waters Corporation, Milford, MA, USA) and fractionated through a BEH C18 column (2.1×150mm, 1.7µm, 130Å) using ACQUITY UPLC® H-Class System (Waters Corporation, Eschborn, Germany) at a flow rate of 250µl/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, 2kV; inlet capillary temperature, 300°C; full-scan automatic gain control (AGC) target, 5×10⁵ ions at 60,000 resolution; scan range, 400–1600m/z; Orbitrap full-scan maximum injection time, 50ms; MS/MS scan AGC target, 2×10⁵ ions at 30,000 resolution; and maximum rejection, 150ms.

Evaluation of clinical outcomes

Just the day before and one month after the surgery, joint functions of patients were evaluated by scores of Visual Analogue Score (VAS), Lysholm, The Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC). All the evaluations were conducted by the same group of doctors.

The analysis of reactions and pathways among proteins

The immune or endocrine system-related proteins which were insignificant abundances between old OA patients and young OA patients were selected out by the ClueGO tool of Cytoscape (v3.7.2) and were analyzed with Reactions and Pathways of Reactome program provided online (<https://reactome.org/>)

Statistical analysis

The proteomic raw data was initially processed with Proteome Discovery 1.4 (Thermo Fisher Scientific, Waltham, MA, USA). KOBAS 3.0 (<http://kobas.cbi.pku.edu.cn/>) [12], a web server for gene/protein functional annotation and functional gene set enrichment, was used for pathway and Gene Ontology functional category enrichment analysis. The false discovery rate (FDR) was calculated by the method described by Benjamin. Significant results were determined based on FDR-adjusted p-value ≤ 0.05. SPSS version 17.0 (SPSS Inc., Chicago, IL) was used for the data analysis, and *P* value < 0.05 was considered as statistically significant. The data of BMI, Mean age, VAS, Lysholm, WOMAC scores were shown as the mean ± standard deviation. The student's t-test was applied for comparing the means of two independent groups and one-way ANOVA analysis was used to compare the means among three groups. Pearson's correlation and linear regression analysis were used to evaluate the correlation between the concentration of estrogen and the VAS, Lysholm as well as WOMAC scores.

Results

The effects of aging and menopause on the clinical outcomes of knee OA and injury women

The basic information of the participants whose ST samples were analyzed as well as the estrogen levels, the pre-surgery and post-surgery evaluated scores were summarized in **Table 1**. Aging, menopause, or estrogen levels were essential factors that would result in a change of post-surgery function outcomes. Even though all participants got a better score after a month of operative therapy, young patients exhibited more agreeable outcomes compared to the aging ones regardless of menopause and disease types ($FDR < 0.05$); on the other hand, premenopausal ladies also enjoyed more satisfying function outcomes than menopausal ones ($FDR < 0.05$). However, no differences in post-surgery function recovery were detected comparing women with higher levels of estrogen to women whose estrogen levels were much lower.

Characteristics of the proteome profile of ST from knee OA and knee injury female patients

Around 4000 proteins were detected in the proteome and finally, 3465 proteins were used for the analysis after quantifying ($FDR < 0.01$). The proteins were composed of different cellular components and were involved in various biological processes (**Fig.1**), according to GO and KEGG analysis.

Proteins profile in ST from OA and knee injury female patients

Our results displayed that ST protein expression patterns were affected by age, menopause, and disease types which were grouped into knee OA group and knee injury group. The average counts of the top 20 candidate proteins which would be most affected by age, menopause, or disease types estrogen levels were listed in **Table 2**.

To better understand whether the protein profiles were affected by estrogen, the estrogen levels were also tested. The differently expressed proteins were as shown in ($FDR < 0.01$) **Table 2**. The relationship between estrogen levels and protein profiles was identified by Pearson correlation analysis. Yet, no significant correlation in protein expression patterns and estrogen levels was detected.

The protein abundances of ST were affected by aging. The protein profiles varied: some proteins were upregulated and the expression levels of some proteins decreased from ST in young female OA and knee injury patients while compared to aging participants (**Fig.2a and b**).

The protein profiles were also influenced by menopause. In comparisons of menopausal knee OA and knee injury patients to the premenopausal ones, some protein levels such as DCTN5, AKR7A2, and NHP2 were significantly increased, while proteins like HDGFL3, MYCBP2 and PRS15 were markedly decreased in ST samples (**Fig.2c**).

Interestingly, the protein profiles of ST from female knee OA patients also differed from those of knee injury women (**Fig.2d**). Female knee OA patients exhibited a higher level of proteins such as TRADD, CTSZ and LZIC; yet a lower level of proteins such as ZFYVE26, LRRC25 and QPRT compared with the knee injury ones.

Network analysis of candidate proteins

Candidate proteins that would be affected by aging, menopause, disease types and estrogen levels were further analyzed by network analysis. The protein-protein interaction network was displayed in **Fig.3**: candidate proteins that mostly affected by aging, as well as their interacted partners, were showed in **Fig.3a**, and then the candidate molecules which significantly influenced by menopause (**Fig.3b**), the ones that were correlated with disease types (**Fig.3c**) and estrogen levels (**Fig.3d**).

The reactions and pathways analysis of candidate proteins analyzed by the Reactome program

The ST proteome data were used for further analysis by the Reactome program. Because the development of OA is associated with the immune system and endocrine system, candidate proteins that were indifferent abundances in ST proteome between menopausal and premenopausal participants were analyzed and those related to the immune or endocrine system were selected. Then those selected proteins were analyzed by Reactions and Pathways in the Reactome program. The potential reactions and pathways among these proteins were shown in **Fig.4**, which might provide insight into the mechanism by which menopause contributes to the development of OA.

Discussion

OA is a leading cause of joint pain and disability, affecting a substantial proportion of people worldwide [13]. Therefore, more and more investigators focus on proteomics studies investigating the compeence and potential roles of synovial fluid [14,15,16,17,18]. Animal models of osteoarthritis are also used in recent studies to understand the pathophysiology of OA and develop successful treatment of OA [19,20]. However, the role of ST of the knee joint in the onset and development of OA remains largely unknown. In this study, we explored the relationship between the recovery of OA after surgery, age, menopause and ST proteome data, aiming at providing insights for mechanisms of the onset and development of OA.

As shown in the results above, aging is the key factor that may influence the post-surgery recovery of knee OA or injury patients, but menopause is not associated. No statistical differences were observed while in comparison to participants with different estrogen levels. The reason might be that estrogen levels may vary according to the period of studied women; the whole picture of their regular mean estrogen levels could not be represented by several tests. Namely, our data of the analyzed estrogen levels were not comprehensive. Since those data might not comprehensively reflect the actual estrogen levels, we were unable to figure out whether estrogen contributes to the remaining joint functions or not. Yet, the role of estrogen in OA initiation and development was inconclusive. According to some surveys, estrogen did contribute to the recovery of OA and could markedly reduce the prevalence of OA [21]. *In vivo* data also suggested that estrogen played an anti-inflammation role in OA animal models [22]. By inhibiting the development of joint swelling and reducing the release of inflammatory cytokines, estrogen could effectively relieve much joint pain and improve the joint swelling situation [22]. Therefore, some estrogen drugs were applied for OA treatment. As one of the OA therapy strategies, estrogen drugs were evaluated by many investors, yet the results and conclusions were inconsistent among the clinical surveys [21,23,24]. Such a phenomenon might be a consequence of various causes.

We demonstrated that the protein profiles vary with aging, menopause, as well as disease types. The proteomic data revealed that the protein composition of old OA women differed from those of young OA women. Our data is consistent with the former report [3]. Unfortunately, perplexed by how age affected human life for centuries, scientists are still unable to explain the aging problem. According to the current technology, we cannot explain how aging leads to the difference in protein mass spectrum? We are also not clear whether such differences are a cause of aging or are a consequence of aging.

It is well established that menopause leads to a significant change of expression levels of some special genes. However, to our best knowledge, similar influences caused by estrogen levels in ST have not been well studied. According to our data, there were no remarkable differences in ST protein expression levels between patients with different estrogen levels at comparable ages, which is quite adverse to the results of a significant association between ST protein profile and menopause. This paradox may be the result of the dynamic fluctuation of estrogen levels in premenopausal women. Women with perimenopausal estrogen levels vary greatly, estrogen levels can be very high.

Similarly, it is also reported that compared to normal or ACL patients, the RNA and protein expression levels demonstrated statistical differences in OA patients in samples such as synovial fluid, synovium and peripheral blood [25]. The different expression genes are supposed to contribute to the initiation and OA and have aroused universal concern, which are optical potential biomarkers of joint diseases. For instance, a report suggests that relevant biomarkers of joint disease are identified by metabolomic profiling of synovial fluid [26].

Then the differently expressed proteins in different groups were further analyzed (Fig.3). Although the proteins that might be changed due to aging, menopause and OA were identified, the pathways via which they might contribute to the joint function remaining or degeneration were still unclear. Protein-protein interaction might provide important clues in studying the molecular mechanisms that might be involved.

The reactions and pathways among immune or endocrine-related proteins which varied significantly in old OA women compared to young OA women were analyzed and visualized by the Reactome program (Fig.4), which might provide insight into the molecular mechanism of aging contributing to the development of OA.

Conclusions

In the study, protein expression profiles of synovial tissues from female knee OA and knee injury patients were analyzed. Around 4000 proteins were identified; 3465 proteins were analyzed. Our results revealed that protein expressions in ST were affected by both aging and menopause. Similarly, aging and menopause also influenced the post-surgery joint functional outcomes of these patients. Therefore, aging and menopause might affect the development of OA as well as the post-surgery recovery of the knee joint through changing the protein expression patterns of ST, we supposed. However, the exact effect of the protein components in ST on the occurrence, development, and rehabilitation of OA remains unclear. More studies are needed to further elucidate the influences of aging and menopause on the knee joint and throw light on the mechanism through which ST might affect the functions of the knee joint.

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

Declarations

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Authors' contributions

X Yang and Z Fu collected the samples and follow-up data, QY Ren analyzed and interpreted the data, and wrote the manuscript. S Wang wrote the manuscript. QY Ren and S Wang collected clinical samples. S Yin and J Liu designed the study, analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the ethics committee of Sichuan Academy of Medical Sciences–Sichuan Provincial People's Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Tables

Table 1 Patients characteristics

Variable	Postmenopausal OA women	Premenopausal OA women	Premenopausal knee injury women
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 post-surgery)			
VAS	6.7±0.58/2.0±1.0	6.3±0.6/1.3±0.6	6.5±0.7/1.0±0.1
Lysholm	69.3±1.16/81.3±4.0	65.3±5.0/92.3±2.1	67.0±1.4/90.5±2.1
WOMAC	148.7±7.02/106.3±10.0	116.7±20.8/71.0±3.6	144.0±5.7/69.0±1.4

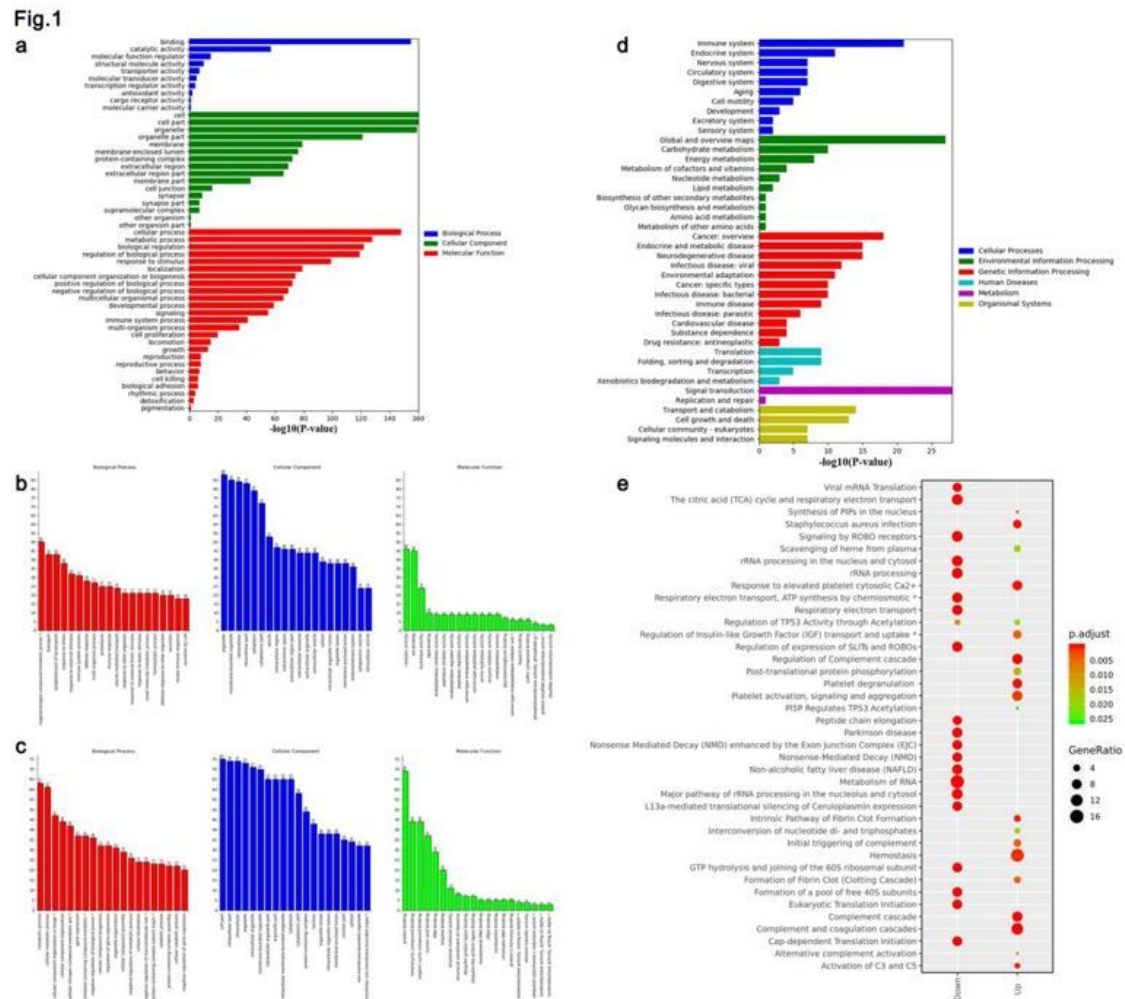
BMI: body mass index; **VAS:** the visual analog scale; **WOMAC:** the Western Ontario and McMaster Universities Osteoarthritis Index.

*Estrogen levels of postmenopausal women are all less than 11.8.

Table 2 The top 20 proteins varied greatly in different groups

young vs old			post- vs pre-menopausal			OA vs knee injury		
Accession	GeneSymbol	Log2(Y/O)	Accession	GeneSymbol	Log2(post/pre)	Accession	GeneSymbol	Log2(OA/injury)
O43488	AKR7A2	0.4269663	Q9BTE1	DCTN5	0.289498664	Q9NXG2	THUMPD1	0.269383267
Q9GZT8	NIF3L1	0.36240516	O43488	AKR7A2	0.409239429	Q96T23	RSF1	0.277527593
P30876	POLR2B	0.26354796	Q9NX24	NHP2	0.302979617	Q9ULT0	TTC7A	0.289193504
P07333	CSF1R	0.493512404	O00160	MYO1F	0.275863833	Q9Y5P4	COL4A3BP	0.290021778
Q9Y2S2	CRYL1	0.477158749	P52630	STAT2	0.294097071	P63172	DYNLT1	0.296420131
Q658P3	STEAP3	0.386304842	Q15257	PTPA	0.283669719	Q9UKK9	NUDT5	0.316220545
Q5SRE7	PHYHD1	0.496614233	P07333	CSF1R	0.443399472	O95858	TSPAN15	0.348805567
P48426	PIP4K2A	0.361906826	O14773	TPP1	0.31591994	Q9NX14	NDUFB11	0.395980419
Q15006	EMC2	0.577473851	Q15006	EMC2	0.682535623	Q13523	PRPF4B	0.521081568
Q9BRG1	VPS25	0.380148739	Q9Y2S2	CRYL1	0.399596671	P07947	YES1	0.6417041
Q00013	MPP1	0.336292433	P55039	DRG2	0.373345031	P41219	PRPH	0.906690157
Q96G23	CERS2	0.602784193	Q9Y3E1	HDGFL3	-0.298723752	Q9Y570	PPME1	-0.502189867
Q01844	EWSR1	0.356185854	O75592	MYCBP2	-0.281459426	O95219	SNX4	-0.440872985
O00487	PSMD14	-0.3046601	P62841	RPS15	-0.371475399	Q15370	ELOB	-0.360115548
O95182	NDUFA7	-0.39374881	Q9UMS6	SYNPO2	-0.441555468	Q13232	NME3	-0.342458488
P53992	SEC24C	0.267462867	Q2TAA2	IAH1	-0.309676687	Q15427	SF3B4	-0.274833517
Q9UK45	LSM7	0.314089197	Q99729	HNRNPAB	-0.325274373	Q13445	TMED1	-0.273530095
Q9NZD2	GLTP	0.335007761	Q13620	CUL4B	-0.381895597	P56545	CTBP2	-0.47964211
P05388	RPLP0	0.286252632	P14621	ACYP2	-0.329073087	Q14938	NFIX	-0.435821005
P61513	RPL37A	-0.60955358	O14495	PLPP3	-0.385721448	Q15628	TRADD	-0.362026664

Figures



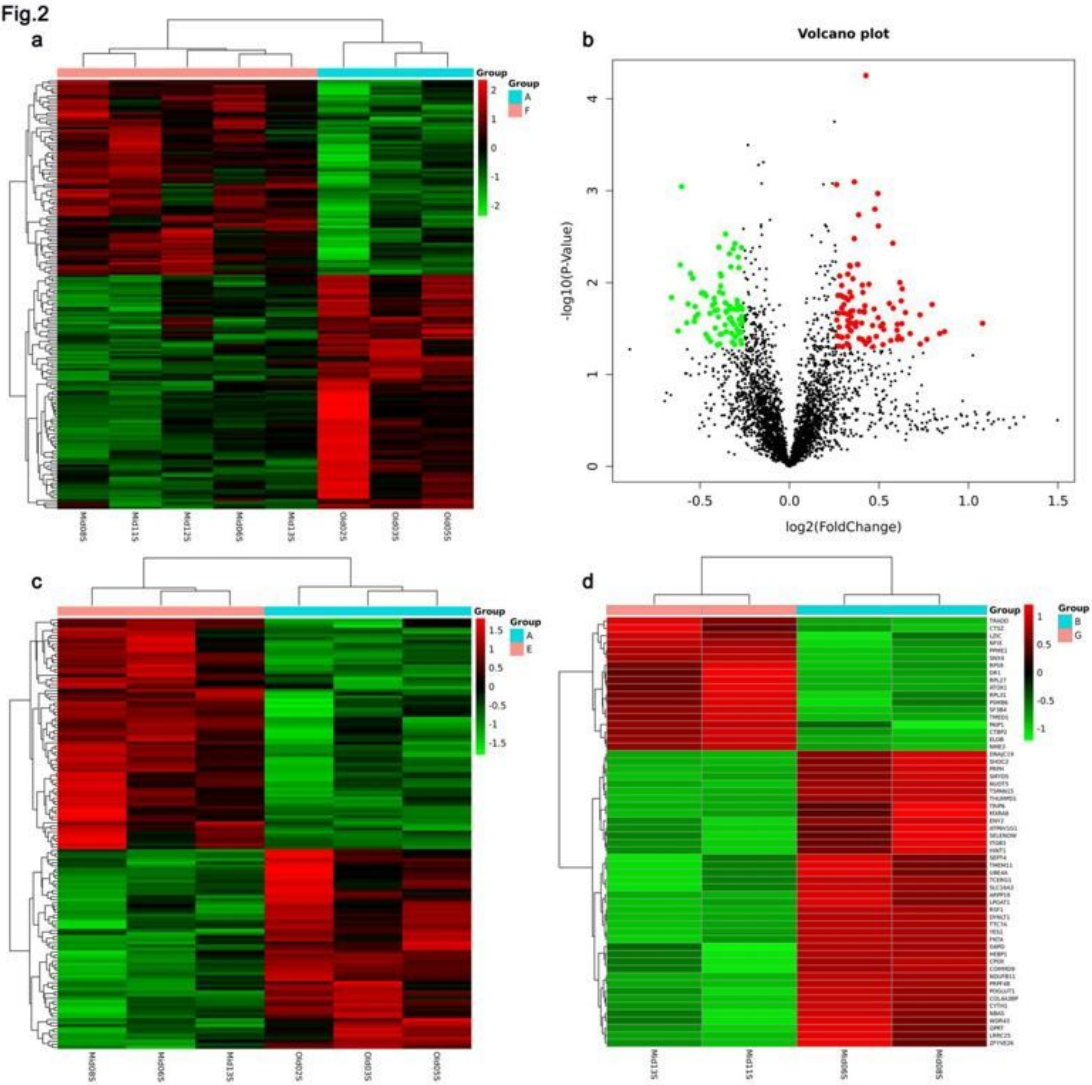


Figure 2

The differently expressed proteins in synovial tissues (ST) samples from different groups. a-b The heatmap (a) and the volcano plots (b) of the differently expressed proteins between aging and young knee osteoarthritis (OA) women. c The heatmap of ST proteins from postmenopausal and menopausal OA women. d The heatmap of ST proteins between menopausal knee OA women and menopausal knee injury women.

Fig.3

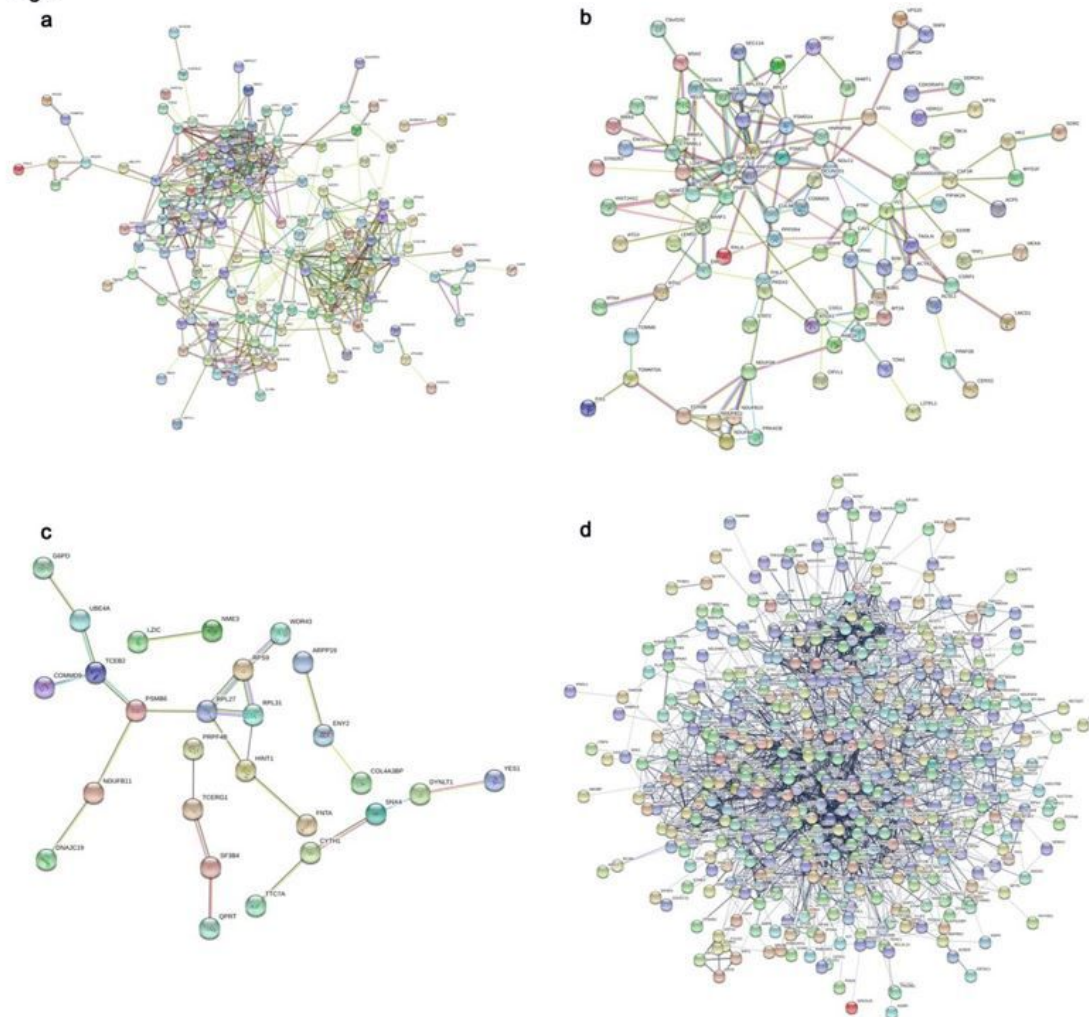


Figure 3

The protein-protein interaction network analyzed by STRING. a The protein-protein interaction network of the proteins most affected by aging from synovial tissues (ST) of knee osteoarthritis (OA) women. b The protein-protein interaction network of the proteins varied greatly with menopause in synovial tissues (ST) of knee osteoarthritis (OA) women. c The protein-protein interaction network of the proteins statistical different between knee OA and injury women. d The protein-protein interaction network of the proteins statistical different in ST samples from OA women with high and low estrogen level.

Fig.4

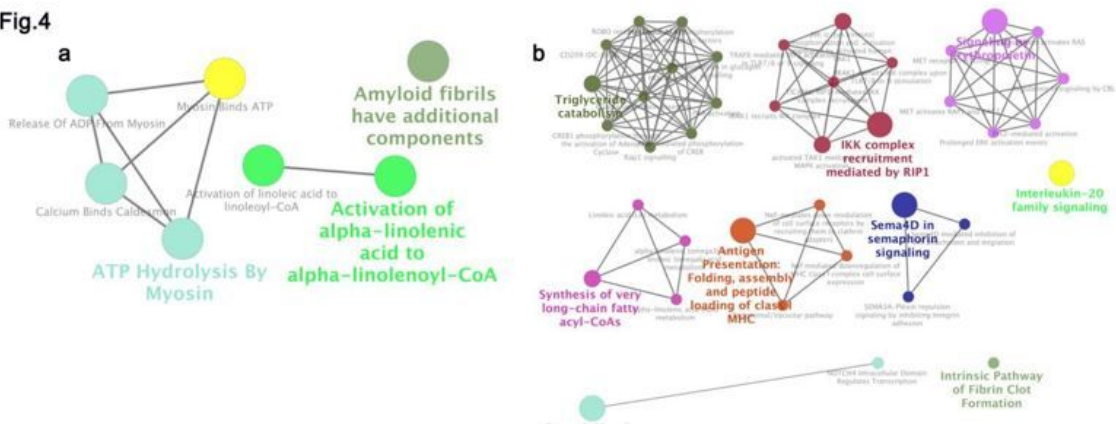


Figure 4

The 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.

Supplementary Files

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

- [SupplementaryMaterial.rar](#)