

Application of integrated omics in aseptic loosening of prostheses after hip replacement

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Abstract. Aseptic loosening (AL) of artificial hip joints is the most common complication following hip replacement surgery. A total of eight patients diagnosed with AL following total hip arthroplasty (THA) undergoing total hip replacement and eight control patients diagnosed with avascular necrosis of femoral head (ANFH) or femoral neck fracture undergoing THA were enrolled. The samples of the AL group were from synovial tissue surrounding the lining/head/neck of the prosthesis, and the samples of the control group were from the synovium in the joint cavity. The present study utilized second-generation high-throughput sequencing and mass spectrometry to detect differentially expressed genes, proteins and metabolites in the samples, as well as Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis. Key genes cytokine receptor-like factor-1 (CRLF1) and glutathione-S transferase μ 1 (GSTM1) expression levels were verified by reverse transcription-quantitative PCR and western blotting. The integrated transcriptomics, proteomics and untargeted metabolomics analyses revealed characteristic metabolite changes (biosynthesis of guanine, L-glycine and adenosine) and decreased CRLF1 and GSTM1 in AL, which were primarily associated with amino acid metabolism and lipid metabolism. In summary, the present study may uncover the underlying mechanisms of AL pathology and provide stable and accurate biomarkers for early warning and diagnosis.

Introduction

Artificial hip replacement surgery is one of the most effective surgical methods in orthopedic treatment of end-stage

hip joint disease. Number of total hip arthroplasties (THAs) in the United States is expected to increase from 49,8000 in 2020 to 1,429,000 in 2040 (1). Despite notable improvements in surgical methods and prosthesis design, aseptic loosening (AL) caused by periprosthetic bone resorption remains a notable cause of hip implant failure and reoperation. Revision surgery can cause physical and mental damage to patients and increase economic pressure on families, society and healthcare systems (2-4). As the life expectancy of patients undergoing joint replacement surgery increases, service life of artificial joints becomes increasingly important. Therefore, prevention and treatment of AL are key to improve the success rate of patients with THA and their quality of life. At present, there are no effective drugs for prevention and treatment of AL in clinical practice.

Metabolites participate in enzymatic chemical reactions which are crucial for cellular function. The metabolome can serve as an important indicator of physiological or pathological status to understand the occurrence and progression of diseases (5-8). Non-targeted metabolomics analysis of intracellular metabolites present during osteoblast differentiation demonstrates glycolysis, nucleotides and lipid metabolism are markedly regulated during osteoblast differentiation (9). Moreover, metabolites associated with oxidative stress are significantly enriched (10). Transcriptomics studies found that pathways related to congenital inflammatory response are the main driving factors for osteolysis in rat models, revealing the mechanism by which mechanical factors lead to implant loosening (11,12). In the present study, metabolomics was used to measure aggregation of all small molecular components of metabolism in AL. A comprehensive multi-omics analysis was conducted on biological samples, changes in metabolites were studied, metabolic properties of AL were determined and metabolic micro-molecular characteristics or biomarkers for AL diagnosis and pathogenesis were investigated.

Materials and methods

Patients and samples. Patients diagnosed with AL after THA (n=8) who underwent revision surgery at the Department of Orthopedics of Henan Provincial People's Hospital (Zhengzhou, China) from May to October 2023 were selected

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as AL group and patients (n=8) diagnosed with avascular necrosis of femoral head (ANFH) or femoral neck fracture who underwent primary THA in the same time period were selected as control group. Inclusion criteria for AL were as follows: i) History of THA surgery; ii) persistent hip pain, limited activity and other symptoms (such as muscle atrophy) after THA; iii) radiological examination (such as X-ray, CT or MRI) shows a radiolucent line or other signs of loosening around the prosthesis and iv) clinical and laboratory examination rule out infection, trauma or other causes of prosthesis loosening (13). Exclusion criteria were as follows: i) Prosthesis loosening caused by postoperative infection, trauma or other diseases (such as bone tumors, systemic lupus erythematosus); ii) postoperative time <6 months (before stable evaluation period) and iii) severe systemic disease that prevents further treatment. Inclusion criteria for controls were as follows: i) Radiological examination (such as X-ray or MRI) shows typical signs of ANFH or femoral neck fracture and ii) clinical examination reveals typical symptoms (ANFH, pain in the groin, limited internal rotation and abduction activities, positive patrick sign; femoral neck fracture: hip pain, limitation of movement, deformity of lower limb, and shortening of affected limb). Exclusion criteria were as follows: i) Hip pain and functional impairment caused by other disease (such as hip infection or tumor); ii) severe systemic inflammatory disease, such as rheumatoid arthritis or systemic lupus erythematosus; iii) severe neurological disease; iv) severe systemic diseases that prevent further treatment and v) mental health issues that prevent treatment and assessment. The samples from patients with AL were collected within 3 months of the onset of loosening symptoms upon completion of diagnosis and revision surgery. The samples of the control group were taken during primary THA surgery. Both AL and control group samples were derived from the surrounding tissue of the liner/head/stem junction of the prosthesis, ensuring consistency in tissue sampling.

The present study was conducted according to the principles of the 1975 Declaration of Helsinki and approved by the Medical Ethics Committee of the Henan Provincial People's Hospital (approval no. 2022-68). All participants provided written informed consent to participate.

Preparation and analysis of metabolomic samples. A total of eight pairs of tissue samples were collected for metabolomics analysis and a 4:1 solution of methanol to water was added to the tissue sample. The samples were ground using a grinder for 6 min (-10°C; 50 Hz), followed by low-temperature ultrasound extraction for 30 min (5°C; 40 kHz). The samples were stored at -20°C for 30 min and centrifuged for 15 min (4°C, 13,000 x g); supernatant was transferred to an injection vial with an internal tube for analysis. The instrument used for liquid chromatography-mass spectrometry (LC-MS) analysis was UHPLC-Q Active system, with an HSST3 chromatographic column (100.0x2.1 mm; internal diameter, 1.8 µm; flow rate of 0.5 ml/min). The sample MS signal was collected in positive and negative ion scanning modes with the following settings: Mass scanning range, 70-1,050 m/z; positive ion voltage 3,500; negative ion voltage 2,800 V; sheath gas, 40 psi; auxiliary heating gas, 10 psi; ion source heating temperature, 400°C; cycle collision energy,

20-60 V; MS1 resolution, 70,000 and MS2 resolution, 17,500 full width at half maxima.

Metabolomic data processing. Raw LC-MS data were imported into Progenesis QI metabolomics processing software (version 2.0, Waters Corporation) for analysis, while MS and MS/MS information was integrated with human metabolome database public metabolic database (hmdb.ca/) and Metlin (metlin.scripps.edu/) and matched with Majorbio database (majorbio.com/). The response intensity of sample MS peaks was normalized using the sum normalization method to obtain the normalized data matrix (14). Variables with relative standard deviation >30% were removed from the quality control samples and log10 logarithmization was performed to obtain the final data matrix for analysis using the R package ropls (version 1.6.2) for principal component analysis (PCA) and orthogonal least squares discriminant analysis (OPLS-DA) (15). Metabolites with variable importance (VIP)>1 and P<0.05 (assessed by unpaired student's t test) obtained from the OPLS-DA model were considered differential metabolites. MetaboAnalyst (Version 5.0) was used for metabolic pathway analysis based on the KEGG and The Small Molecule Pathway Database (SMPDB) databases (16).

Transcriptomic sample processing and analysis. Following tissue grinding as aforementioned, TRIzol (cat. no. 15596018CN, Invitrogen) was added to extract RNA, Oligo dT (cat. no. 18418012, Invitrogen) was used to enrich mRNA, fragmentation buffer was added; mRNA was randomly broken into small fragments of ~300 bp and reverse-transcribed using Hieff NGS ds-cDNA Synthesis Kit (cat. no. 13488ES96, Yeasen); EndRepairMix (cat. no. N203-01/02, Vazyme) was added to supplement the flat end. Next, A base was added at the 3' end to connect the Y-shaped junction. cDNA purification and fragment sorting that utilize beads to selectively bind and isolate the 200-300 bp of DNA fragments were done using sorting kits (cat. no. 12601ES56, Hieff NGS® DNA Selection Beads, Yeasen). The sorted products were used for amplification by PCR using Phusion Hot Start II High-Fidelity DNA Polymerase (cat. no. F565L, Thermo Fisher Scientific). Forward primer: 5'-AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT-3', reverse: 5'-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-3'. Thermo cycling conditions were as follows: Initial denaturation: 98°C for 30 sec to denature the double-stranded DNA, denaturation: 98°C for 15 sec to separate the DNA strands, annealing: 55°C for 30 seconds, elongation: 72°C for 30 sec, 30 cycles, and final extension: 72°C for 5 min. Prepared libraries were performed by VAHTS Universal Plus DNA Library Prep Kit for Illumina (cat. no. ND617-01/02, Vazyme) according to the manual. Qubit 2.0 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to detect the concentration of the library, and the loading concentration of the library were pooled at 10 nM concentration, we performed the 2x150 bp paired-end sequencing (PE150) and an average read depth of 15 million read pairs/library on Illumina NovaSeq X Plus platform (Illumina, Inc.) following the vendor's recommended protocol. Illumina BaseSpace (Version: V5.2.0, illumina.com/software/basespace.html) for base calling and demultiplexing. Trimmomatic (Version:

V0.39, usadellab.org/cms/?page=trimmomatic) for quality trimming of sequence reads. STAR (Version: V2.7.3a, URL: <https://github.com/alexdobin/STAR>) for aligning reads to a reference genome.

Transcriptomic data processing. DESeq2 (Version 1.24.0; bioconductor.org/packages/stats/bioc/DESeq2/) with a screening threshold of $|\log_2FC| \geq 1$ and $P_{adj} < 0.05$ was used to identify differentially expressed genes (DEGs). Functional enrichment analyses included Kyoto Encyclopedia of Genes and Genomes (KEGG; Version 2022.10; genome.jp/kegg/), Gene Ontology (GO; [goatools](https://goatools.org/); Version 0.6.5; files.pythonhosted.org/packages/bb/7b/0c76e3), Reactome (Version 82; reactome.org) and Disease Ontology (DO; disease-ontology.org) enrichment analyses. The screening threshold for determining significant differences in transcript expression between samples was determined by DESeq2, with $P_{adj} < 0.05$. P-value was corrected using the Benjamini-Hochberg method. $P_{adj} < 0.05$ was considered to indicate significant enrichment.

Proteomic sample processing and analysis. The tissue samples were ground as aforementioned to extract protein and concentration was measured using the BCA method. Enzymatic alkylation was performed by adding iodoacetamide (10 mM, room temperature for 30 min) to protein. Adding DL-Dithiothreitol (DTT, 50 mM, room temperature for 15 min) to quenching the reaction to generate stable and specific peptides for mass spectrometry analysis. Enzymatic alkylation was performed on 100 μ g samples; the next day, samples were subjected to tandem mass tag labeling and mixing, mixed with an equal amount of labeled products in a tube, dried with a vacuum concentrator (30°C, 20 min) and the peptide samples were dissolved in Ultra Performance Liquid Chromatography buffer (Waters Corporation). Next, high-pH liquid-phase separation was performed using a reverse-phase C18 column and the two-dimensional Easy-nLC1200 result was analyzed by using a QExactive (Thermo Fisher Scientific, Inc.) mass spectrometer. The peptide segments were dissolved in MS loading buffer (Thermo Fisher Scientific, Inc.) and subjected to separation in a C18 chromatography column (35°C, 5 μ l, 75 μ Mx25 cm; Thermo Fisher Scientific, Inc.) for 120 min at a flow rate of 300 μ l/min. The process was based on EASY-nLC liquid-phase gradient elution [phase A, 2% acetonitrile (with 0.1% formic acid) and B, 80% acetonitrile (with 0.1% formic acid)] with the following settings: 0-1 min, 0-5% B; 1-63 min, 5-23% B; 63-88 min, 23-48% B; 88-89 min, 48-100% B and 89-95 min, 100% B. MS and MS/MS modes were switched automatically for collection, with MS resolutions of 70 and 35 K, respectively. With each MS full scan (m/z, 350-1,300), the top 20 parent ions were selected for secondary fragmentation, with dynamic exclusion time of 18 sec.

Proteomic data processing. The original files were analyzed by using ProteomeDiscoverer™ (version 2.2; Thermo Fisher Scientific, Inc.). The false discovery rate for peptide identification during the search process was ≤ 0.01 . The t test function in R software (search.r-project.org/CRAN/refmans/DACF/html/lw.t.test.html; version 1.6.2) was used to calculate the significance of the inter-sample

differences, as well as the fold-change (FC) of the inter-group differences. The screening criteria for significantly differentially expressed proteins were $P < 0.05$ and $FC > 1.2$ for up- and $FC < 0.83$ for downregulated proteins. Functional annotation and metabolic pathway analysis were performed on all differentially expressed proteins. GO enrichment analysis was performed using Goatools (Version no. 1.4.4; pypi.org/project/goatools/) and Fisher's exact test. Based on Meiji's independently developed process, KEGG pathway enrichment analysis was performed (17). $P_{adj} < 0.05$ was considered to indicate significant enrichment.

Comprehensive analysis. Using Cytoscape (version 3.9.1; js.cytoscape.org/), a network of genes, proteins and metabolic compounds was constructed to identify pathways significantly enriched according to DEGs and reveal potential regulatory mechanisms between genes and metabolites. Differential metabolites and DEG expression data between the AL and control group were imported into Cytoscape to assess genetic and metabolic changes in AL, as well as the potential mechanisms of metabolism.

Bioinformatics analyses. DESeq2 (Version 1.24.0; bioconductor.org/packages/stats/bioc/DESeq2/) was used for differential gene analysis. KEGG (Version 2022.10; genome.jp/kegg/), GO ([goatools](https://goatools.org/); Version 0.6.5; files.pythonhosted.org/packages/bb/7b/0c76e3) and Reactome databases (Version 82; reactome.org/) were used to determine signal transduction pathways related to the DEGs. DO database (<https://disease-ontology.org/>) was used to determine human diseases associated with DEGs, while the GO and KEGG databases were used for protein functional annotation and functional enrichment. R software (Version 1.6.2) was used for differential protein analysis in sample tissues. MultiLoc2 (Version 2.0) was used for subcellular localization analysis (18). Differential metabolite analysis was performed with ropls (master.bioconductor.org/packages/stats/bioc/ropls/; R package; Version 1.6.2) and multivariate statistics with scipy (<https://www.scipy.org/>; Python; Version 1.0.0) based on KEGG pathway enrichment results of the human metabolism, metabolic disease and metabolite signaling pathways associated with differential metabolite enrichment.

Reverse transcription-quantitative (RT-q)PCR. Total RNA from AL samples and controls was isolated using TRIzol (Thermo Fisher Scientific, Inc.). RNA was subjected to phenol-chloroform extraction for purification. The quantity and quality of the purified RNA were assessed by measuring the absorbance at 260/280 nm (acceptable ratio ≤ 1.8 and ≥ 2.2) using Microplate Reader (Thermo Fisher Scientific, Inc.). cDNA was synthesized using HiScript II RT SuperMix (Vazyme Biotech Co., Ltd.) at 37°C for 15 min and 85°C for 5 sec and maintained at 4°C. RT-qPCR was conducted with AceTaq DNA Polymerase (Vazyme Biotech Co., Ltd.) as follows: Initial denaturation at 95°C for 1 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Each transcript concentration was normalized to the level of GAPDH using the $2^{-\Delta\Delta C_q}$ method (19). The primer sequences were as follows: GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'; cytokine

Table I. Patient and control demographics.

Characteristic	Control	AL	P-value
Sex, male/female	2/6	2/6	>0.999
Mean age, years	61.88±2.29	60.75±3.62	0.797
Mean BMI	25.29±1.05	23.77±1.35	0.388
Operative site, left /right	5/3	5/3	>0.999
Type of surgery	Primary total hip arthroplasty	Revision total hip arthroplasty	
Drinking history, yes/no	2/6	1/7	0.521
Smoking history, yes/no	2/6	1/7	0.521
Preoperative diagnosis, avascular necrosis of femoral head/fracture of neck of femur	5/3	6/2	0.589

receptor-like factor-1 (CRLF1) forward, 5'-CTCTCCCGT GTACTCAACGC-3' and reverse, 5'-GGGCAGGCCAACATA GAGG-3' and glutathione-S transferase μ 1 (GSTM1) forward, 5'-GCCCATGATACTGGGGTACTG-3' and reverse, 5'-GGG CAGATTGGGAAAGTCCA-3'.

Western blotting. Samples from patients with AL and controls were collected and lysed in RIPA buffer (Merck KGaA) on ice for 30 min. Protein concentration was determined using the BCA method. A total of 20 μ g/lane protein samples were separated by 10% SDS-PAGE and transferred onto PVDF membranes (MilliporeSigma). The membrane was blocked with 5% skimmed milk at room temperature for 2 h. Subsequently, the membrane was incubated overnight at 4°C with primary antibodies targeting CRLF1 (1:1,000; 43 kDa; cat. no. bs-8663R; Beijing Biosynthesis Biotechnology Co., Ltd.), GSTM1 (1:2,000; 27 kDa; cat. no. 12412-1-AP; Wuhan Sanying Biotechnology) and GAPDH (1:10,000; 36 kDa; cat. no. HRP-60004; Wuhan Sanying Biotechnology). Horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. no. I1904-65C; Shanghai Univ Biotechnology Co., Ltd.) were incubated at room temperature for 2 h. Signal analysis was performed using enhanced chemiluminescence reagent (cat. no. BL520A, Biosharp) and an image analyzer (Bio-Rad Laboratories) to detect protein expression levels, and Image Lab software (Bio-Rad Laboratories; Version 6.1). The intensity of each band was quantified using AlphaEaseFC software.

Statistical analysis. Data were analyzed using GraphPad Prism (version 6.01; Dotmatics). Continuous variables that conform to normal distribution are presented as the mean $\pm$ standard deviation of ≥ 3 independent experimental repeats and were tested using unpaired Student's t-test. Categorical variables were tested using χ^2 test. Pearson correlation analysis was performed between CRLF1, GSTM1 and differential metabolites. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinical characteristics of patients. All patients presented with unilateral onset of AL and had undergone unilateral surgery. Of patients with AL who had undergone revision

surgery, reasons for the initial total hip replacement included ANFH in six cases and femoral neck fracture in two cases. The friction interface of the initial replacement surgery in the AL group was ceramic on polyethylene in six cases and metal on polyethylene in two cases. The initial prosthesis fixation types in the AL group were cementless in seven cases and cemented in one case; in the latter, the femoral stem was cemented and the acetabular cup cementless, the acetabular cup did not loosen, but the femoral stem prosthesis did. In all cases in the AL group, prosthesis failure due to infection was excluded. AL group consisted of six females and two males, with a mean age of 60.75±3.62 years. The average duration from the initial replacement surgery to the revision surgery in the AL group was 109.5±62.99 months. In the AL group, there were three cases of isolated acetabular cup loosening, one case with isolated femoral stem loosening and four cases with the loosening of both the acetabular cup and femoral stem.

In the control group, reasons for surgery included ANFH in five cases and femoral neck fracture in three cases. The control group consisted of six females and two males, with an average age of 61.88±2.29 years. There were no significant differences in sex ratio or the average age between the two groups (Table I).

Transcriptomics. There were 454 DEGs in the AL vs. control groups (Table SI), 33 of which were up- and 421 were downregulated. Triadin, which is associated with muscle contraction (20), was the most significantly downregulated gene in patients with AL. PRKY gene was significantly upregulated in the AL group (Fig. 1A). To determine molecular functions (MFs) affected by differential gene expression, the KEGG database was used. A total of 17 enriched KEGG pathways were identified, including 'cardiac muscle contraction', 'hypertrophic cardiopathy' and 'dilated cardiopathy' (Fig. 1B). By mapping DEGs to GO database for analysis, it was revealed that there may be an association between AL and genes involved in the regulation of the 'troponin complex', 'transition between fast and slow fiber', 'cellular response to purine-containing compound', 'response to stimulus involved in regulation of muscle adaptation', 'telethonin binding' and 'FATZ binding' (Fig. 1C). Reactome database revealed significant changes in reactions and biological pathways such as 'muscle contraction', 'transport of small molecules' (Fig. 1D).

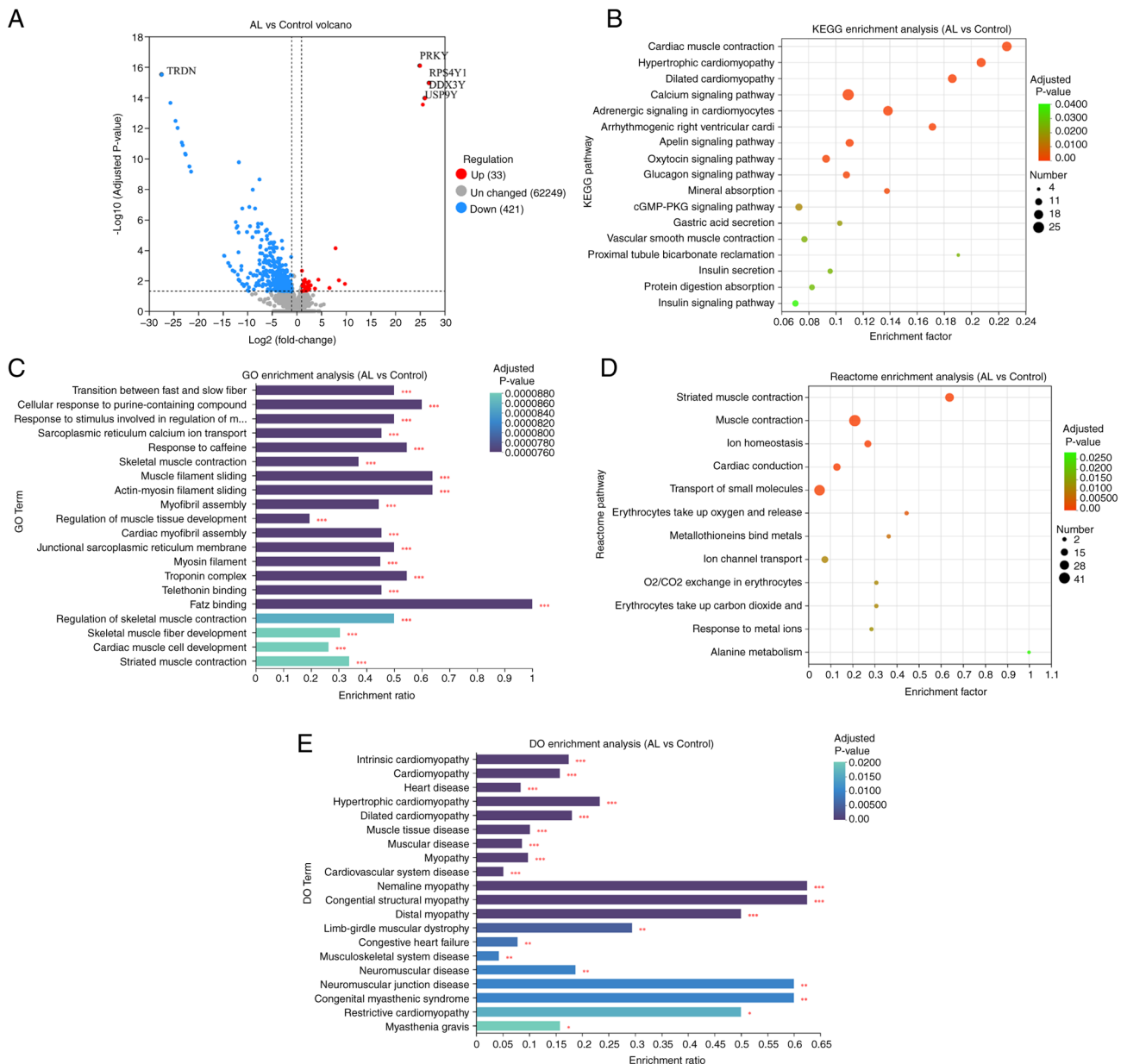


Figure 1. Transcriptomics analysis of AL and control tissue. (A) Volcano diagram of differentially expressed genes. (B) KEGG and (C) GO enrichment analysis. (D) Reactome and (E) DO enrichment analysis of transcriptome data. AL, aseptic loosening; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; DO, Disease Ontology. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

DO showed enrichment of genes associated with diseases such as 'intrinsic cardiopathy', 'cardiomyopathy' and 'heart disease' (Fig. 1E).

Proteomics. Between AL and control, there were 133 differentially expressed proteins, 107 of which were up- and 26 were downregulated (Fig. 2A). The most significant downregulation in the AL group was cyclin-dependent kinase 9 protein, which is associated with osteoclastogenesis and bone resorption activity (21). The expression of proline rich coiled-coil 2C protein was significantly upregulated (Fig. 2A). KEGG enrichment analysis indicated nine significantly enriched KEGG pathways, including 'NOD-like receptor signaling pathway', 'osteoclast differentiation', and 'Shigellosis'. (Fig. 2B). In GO, 'macromolecule biosynthetic process', 'cytoplasmic ribonucleoprotein granule', and 'antigen processing and presentation'

were enriched (Fig. 2C). Subcellular localization analysis elucidates the specific cellular localization of differential proteins, which is closely related to protein function (22). The differentially expressed proteins were primarily located in the cytoplasmic, nuclear and mitochondrial regions (Fig. 2D).

Metabolomics. PCA (Fig. 3A) and OPLS-DA (Fig. 3B) showed significant separation and metabolic changes. According to VIP > 1.5 , 113 significant differential metabolites were screened, including 95 up- and 18 downregulated. Dextrophan O-glucuronide was significantly downregulated and caprylic acid was significantly upregulated, with the highest VIP value of 4.32 (Fig. 3C and D). KEGG annotation showed differential compounds were primarily associated with metabolism (Fig. 3E), with significant enrichment of 'glycine, serine and threonine metabolism', 'phenylalanine metabolism', 'glyoxylate

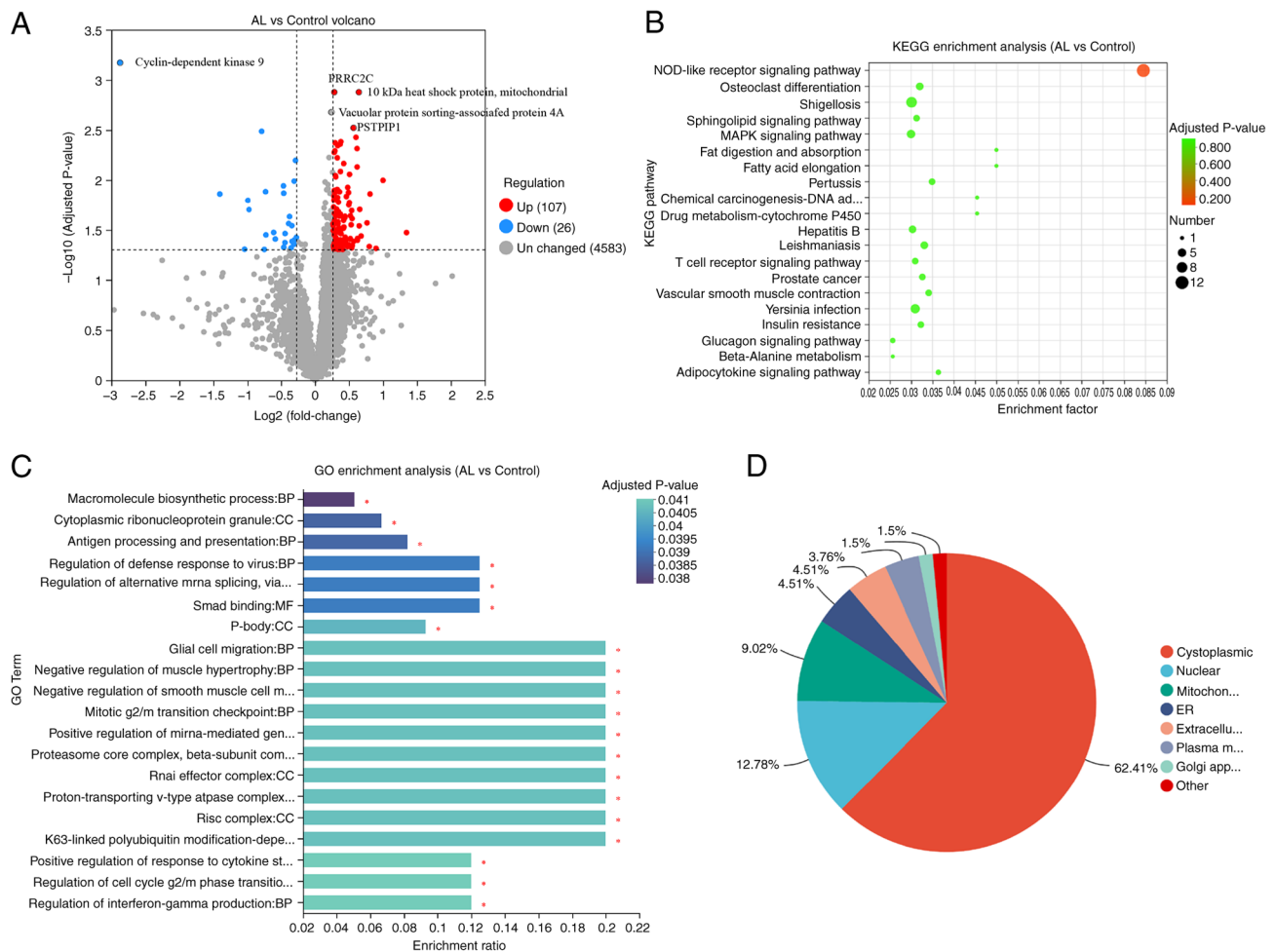


Figure 2. Proteomics analysis of AL and control tissue. (A) Volcano plot of differentially expressed proteins. (B) KEGG enrichment analysis. (C) GO enrichment and (D) subcellular localization analysis of proteomic data. AL, aseptic loosening; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology. * $P < 0.05$.

and dicarboxylate metabolism', 'tyrosine metabolism' and 'arginine and proline metabolism' (Fig. 3F). Differential expression of GSTM1 and CRLF1 was consistent at the mRNA and protein levels (Fig. 4A). Pearson correlation analysis between CRLF1 and GSTM1 genes and differential metabolites showed that these genes were associated with changes in 44 metabolites (Fig. 4B). SMPDB (Fig. 4C) and KEGG enrichment analysis (Fig. 4D) showed that CRLF1 and GSTM1 affected 'pyruvate metabolism', 'citrate cycle (TCA cycle)', 'tyrosine metabolism', 'purine metabolism', 'valine, leucine and isoleucine degradation', 'arginine and proline metabolism', 'phenylalanine, tyrosine and tryptophan biosynthesis'. Corresponding metabolites of associated pathways, such as malic acid, guanine, L-tyrosine, niacinamide, L-valine, L-proline, L-isoleucine and L-phenylalanine were increased (Fig. 4E).

Integration of transcriptomics, proteomics and metabolomics. Pathway analysis was conducted at the transcriptional, protein and metabolite levels and KEGG enrichment revealed common pathways regulated at transcriptional, protein and metabolite levels, with 24 pathways in the AL group (Fig. 5). The 'arginine and proline metabolism', 'purine metabolism' and 'glutathione metabolic pathways' were regulated at the transcriptional, protein and metabolite levels in AL. The

metabolites guanosine 3'-monophosphate, deoxyguanylic acid, adenosine 3'-monophosphate, guanine, L-glycine and adenosine were significantly overexpressed in the AL group, participating in the 'purine metabolic pathway' and affecting expression levels of the guanine deaminase (GDA), Adenylosuccinate Synthase 1 (ADSS1), Adenosine Monophosphate Deaminase 1 (AMPD1), Adenylate Kinase 1 (AK1), Adenylate Cyclase 2 (ADCY2) and 5'-Nucleotidase, Cytosolic IA (NT5C1A) genes and the 5'-Nucleotidase Ecto (NT5E) and Deoxyguanosine Kinase (DGUOK) proteins. The 'arginine and proline metabolic pathway' is a key pathway in AL, in which the metabolic levels of L-pyrroline-2-carboxylic acid and protein expression of Leucine Aminopeptidase 3 (LAP3) was increased, and gene expression of the nitric oxide synthase 1 (NOS1), Creatine Kinase M-Type (CKM), 4-Hydroxy-2-Oxoglutarate Aldolase 1 (HOGA1), Carnosine Synthase 1 (CARNS1) and Creatine Kinase, Mitochondrial 2 (CKMT2) genes were downregulated. The 'glutathione metabolic pathway' is also one of the important pathways in AL, in which the expression level of the L-glycine metabolite was significantly increased, the gene and protein expression levels of GSTM1 were significantly reduced, the LAP3 protein expression level was significantly increased, and the gene expression level of MGST1 was significantly reduced (Fig. 5).

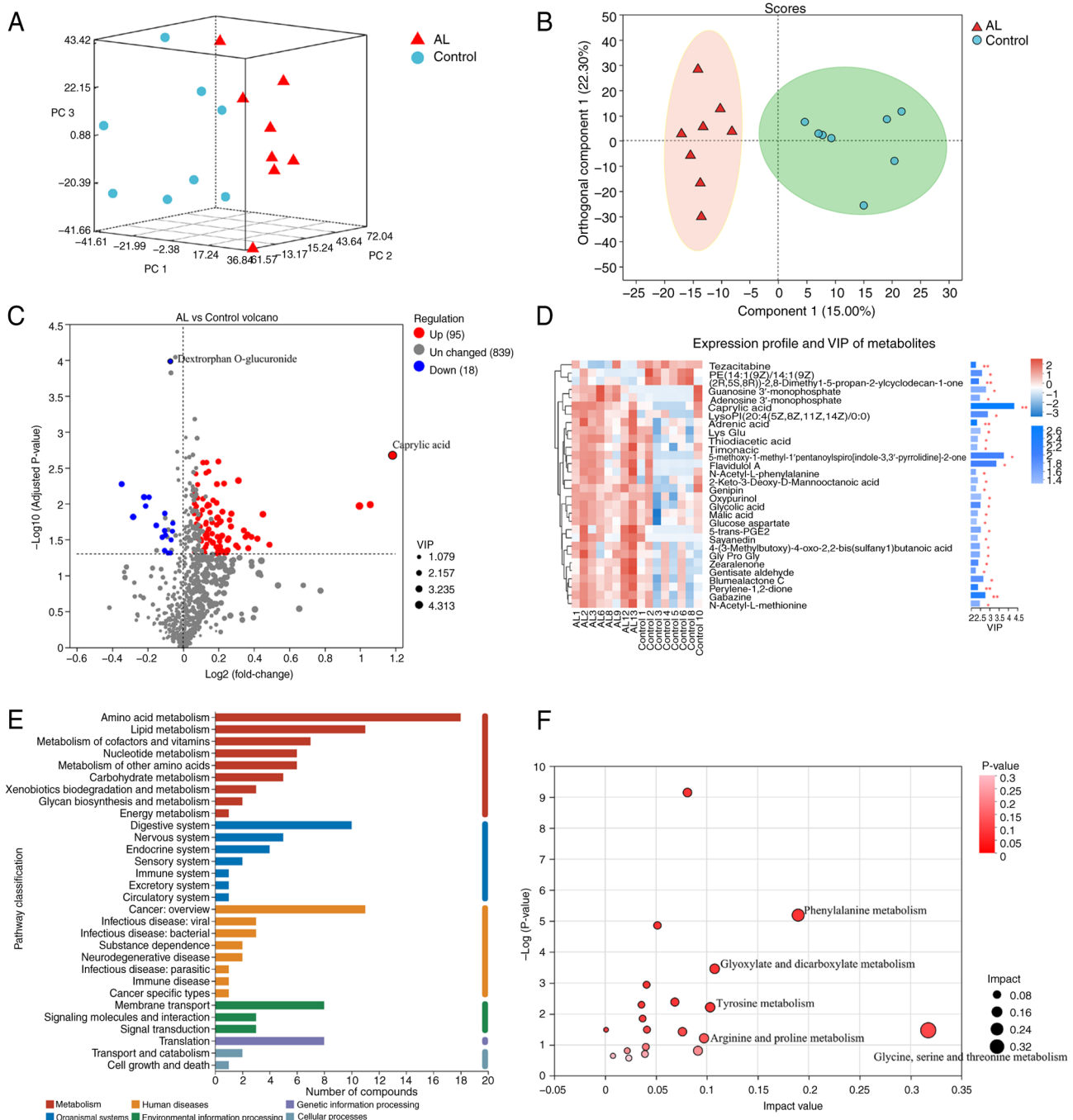


Figure 3. Metabolomics analysis of AL and control tissue. (A) PC and (B) orthogonal partial least squares discriminant analysis. (C) Volcano diagram of differentially expressed metabolites. (D) VIP analysis of metabolic data. KEGG (E) annotation and (F) enrichment analysis. KEGG, Kyoto Encyclopedia of Genes and Genomes; AL, aseptic loosening; PC, principal component; VIP, variable importance. * $P < 0.05$, ** $P < 0.01$

DEG verification. DEG verification was conducted using RT-qPCR and western blotting to measure mRNA and protein expression levels of CRLF1 and GSTM1 in tissue samples. The results showed that, compared with the control group, the mRNA expression of CRLF1 and GSTM1 in AL (Fig. 6A and B), as well as protein expression (Fig. 6C-E), was significantly decreased.

Discussion

AL of prostheses is the primary cause of revision surgery, and its occurrence and development are associated with

metabolic disorders of bone formation and dissolution around joint prostheses, as well as aseptic inflammation induced by prosthesis wear particles (such as metal and polyethylene particles) (23). In the present study, transcriptome, proteomic and non-targeted metabolomic data were analyzed in synovial tissue and a combined multi-omics analysis was conducted to reveal changes in metabolites and potential pathogenesis in AL, providing a novel perspective for the pathogenesis and potential diagnosis.

Driven by advances in high-throughput technology, transcriptomics, proteomics and metabolomics have clinical application and biomarkers can be used to improve accuracy,

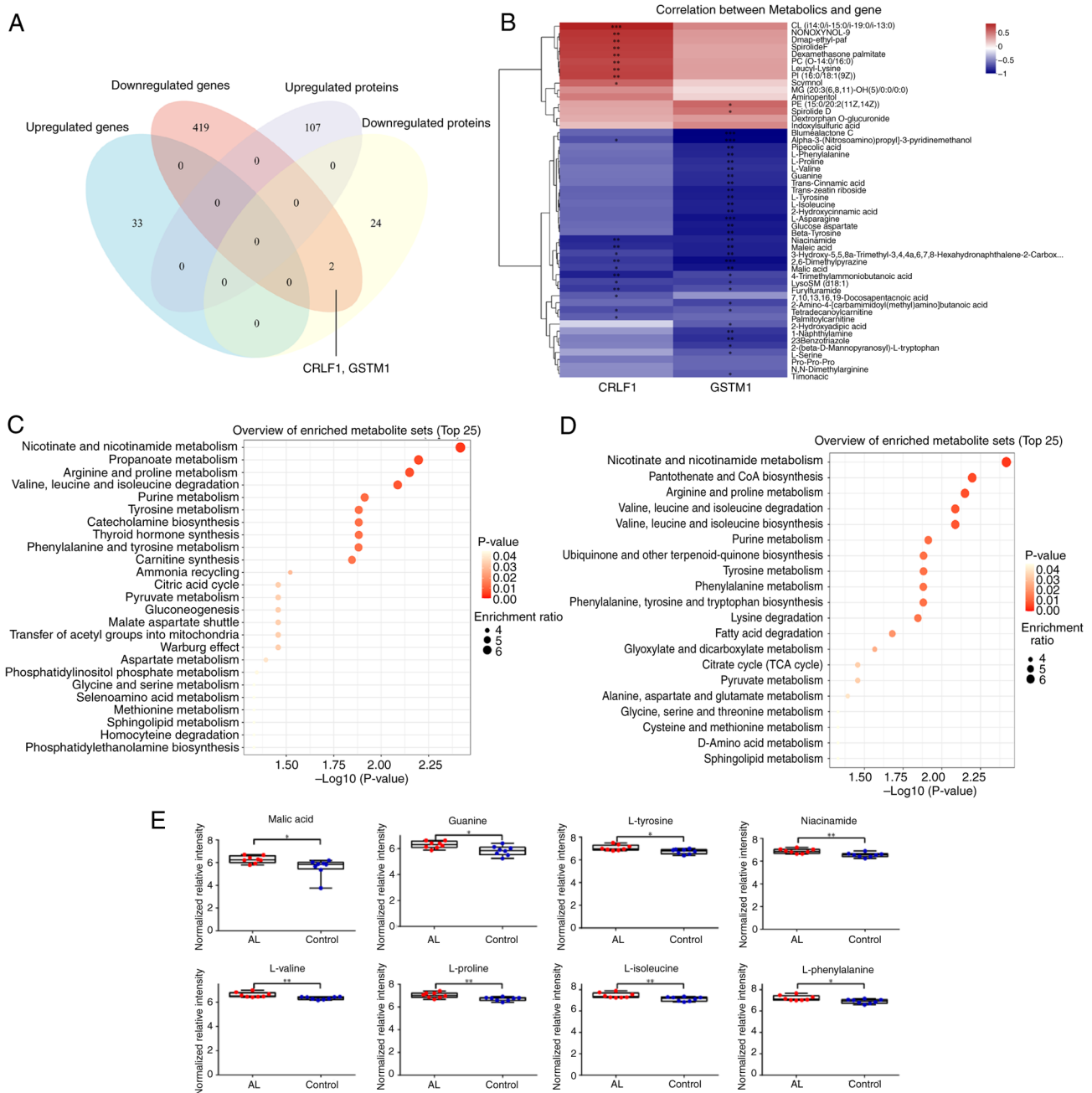


Figure 4. Metabolomics analysis of differentially expressed genes in transcriptomics and proteomics. (A) Differentially expressed genes CRLF1 and GSTM1 in transcriptomics and proteomics. (B) Correlation analysis between CRLF1, GSTM1 and differential metabolites. (C) Small Molecule Pathway Database enrichment analysis of metabolic data. (D) Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of metabolic data. (E) Significantly differentially expressed metabolites. CRLF1, cytokine receptor-like factor-1; GSTM1, glutathione-S transferase μ 1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

enhance diagnosis and decrease errors (24). Functionally, the transcriptome encompasses all RNA present in cells; although a large portion of it is not translated into proteins, it serves a role in determining cell phenotype and has clinical value in clinical diagnosis (25). Proteomics can complement other 'omics' techniques, such as genomics and transcriptomics, to identify the structure and function of specific proteins (26). By contrast, metabolomics is primarily used to determine small-molecule fingerprints of cellular processes (27). Metabolites are the final downstream products of protein translation, gene transcription or cellular disturbances in the proteome, genome, or transcriptome. As the final product of

cell regulatory processes, they are considered the ultimate response of biological systems to metabolic disorders and pathophysiological changes (28). However, the proteome and metabolome are connected. The protein expression affects the metabolic profile and concentration of metabolites in turn affects protein expression (29). Therefore, integrated omics may provide insights into biological systems and mechanisms.

The present study identified CRLF1 and GSTM1 as potential biomarkers for AL. CRLF1 is a soluble type I cytokine receptor that serves an important role in the immune system and fetal development (30). It is upregulated by proinflammatory cytokines such as TNF- α , IL-6 and IFN- γ , indicating that

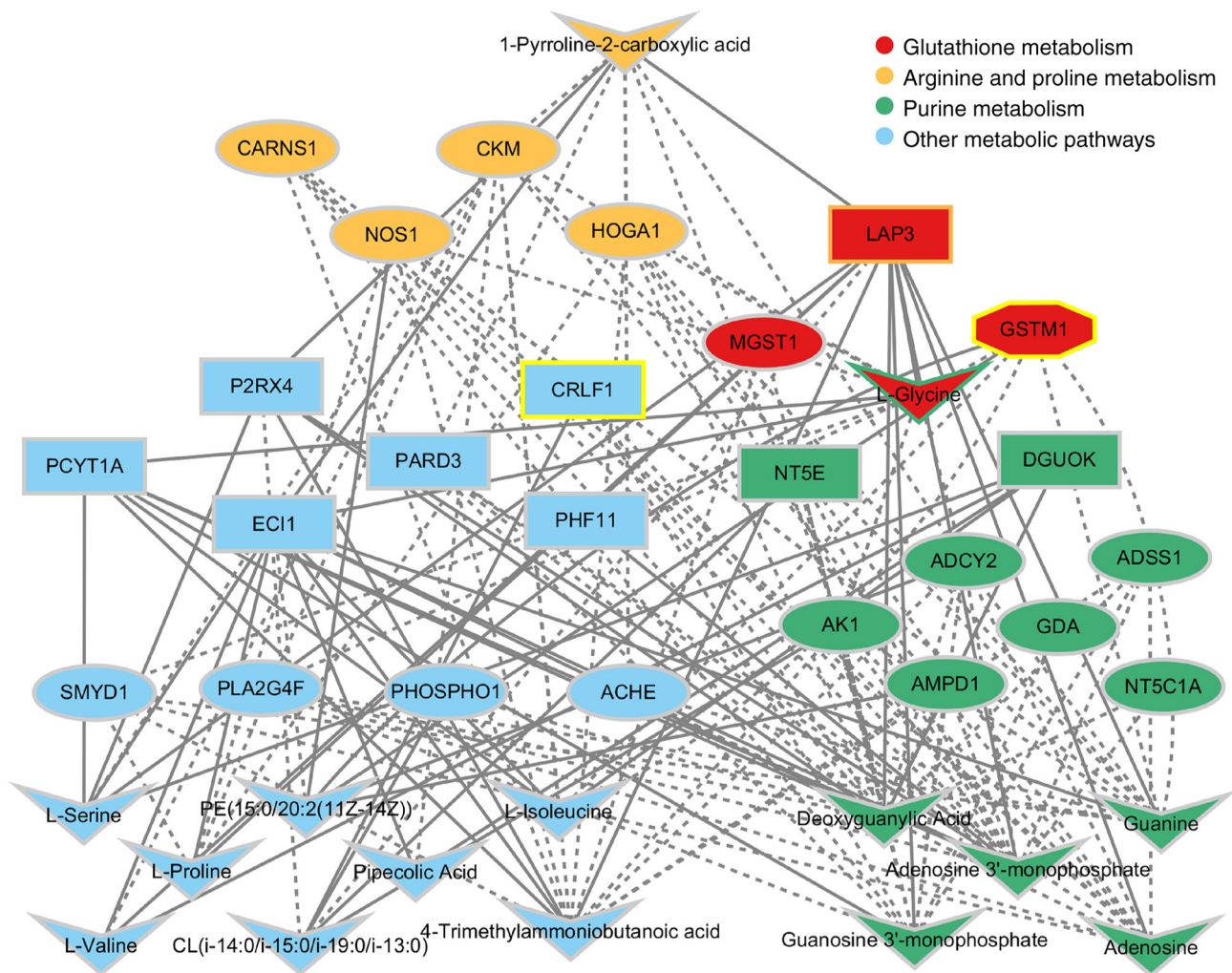


Figure 5. Interactions between DEGs, proteins and metabolites. Ellipses, rectangles, arrowheads and octagons represent DEGs and differentially expressed proteins, metabolites and proteins with DEGs, respectively. The solid and the dashed lines represent positive and a negative correlation, respectively. Yellow indicates key nodes. DEG, differentially expressed gene.

human CRLF1 may participate in immune system regulation during the inflammatory response (31). As this protein is expressed at high levels in damaged human knee osteoarthritis cartilage and participates in TGF- β downregulation, it may serve as a biomarker for osteoarthritis (32,33). The transcription and protein levels of CRLF1 were significantly decreased in AL synovial tissue, suggesting that CRLF1 may be involved in wear particle-induced aseptic inflammation in AL. GSTM1, belonging to the glutathione S-transferase superfamily, is involved in the metabolism and detoxification of reactive oxygen species (ROS) and carcinogens (34). It serves a key role in determining disease susceptibility, with research showing that ineffective variants of GSTM1 are associated with increased risk of ovarian cancer (35). Cytochrome P450 family 1 subfamily a member 1 and GSTM1 polymorphisms are genetic risk factors in patients with bone tumors and allele variations in these genes increase risk of bone tumor occurrence (36). Given the association between GSTM1 and glutathione S-transferase 01 genes and bone mineral density, these genes may be used as candidates for studying the genetics of osteoporosis (37). Glutathione metabolism and ferroptosis serve important roles in normal differentiation of osteoblasts

and senile osteoporosis. GSTM1 and transferrin receptor (TFRC) are key genes in this process, involved in decreasing ROS levels in senile osteoporotic osteoblasts (38). GSTM1 is a phase II enzyme of the glutathione-S-transferase family that protects cells by catalyzing conjugation of hazardous chemicals to reduced glutathione (GSH) (39). TFRC encodes transferrin receptor protein 1 (TFR1) in humans, which controls the levels of intracellular iron levels (40). TFR1 imports iron from the extracellular environment into cells, contributing to the cellular iron pool, and serves a key role in ferroptosis (40). Kinov *et al* (41) demonstrated that the occurrence of AL is associated with high oxidative stress, GSH/oxidized glutathione ratio of loose hip prostheses is lower than that of stable hip prostheses, suggesting that high oxidative stress may serve a key role in AL. Dong *et al* (42) showed that DNA methylation-mediated glutathione peroxidase 4 transcriptional suppression and osteoblast ferroptosis can promote osteolysis induced by titanium particles. Xu *et al* (43) confirmed that regulating osteoblast ferroptosis via NF-E2-related factor 2 (Nrf2)/antioxidant response element signaling induces peri-implant osteolysis (43). Here, GSTM1 was significantly downregulated at both transcriptional and protein levels in

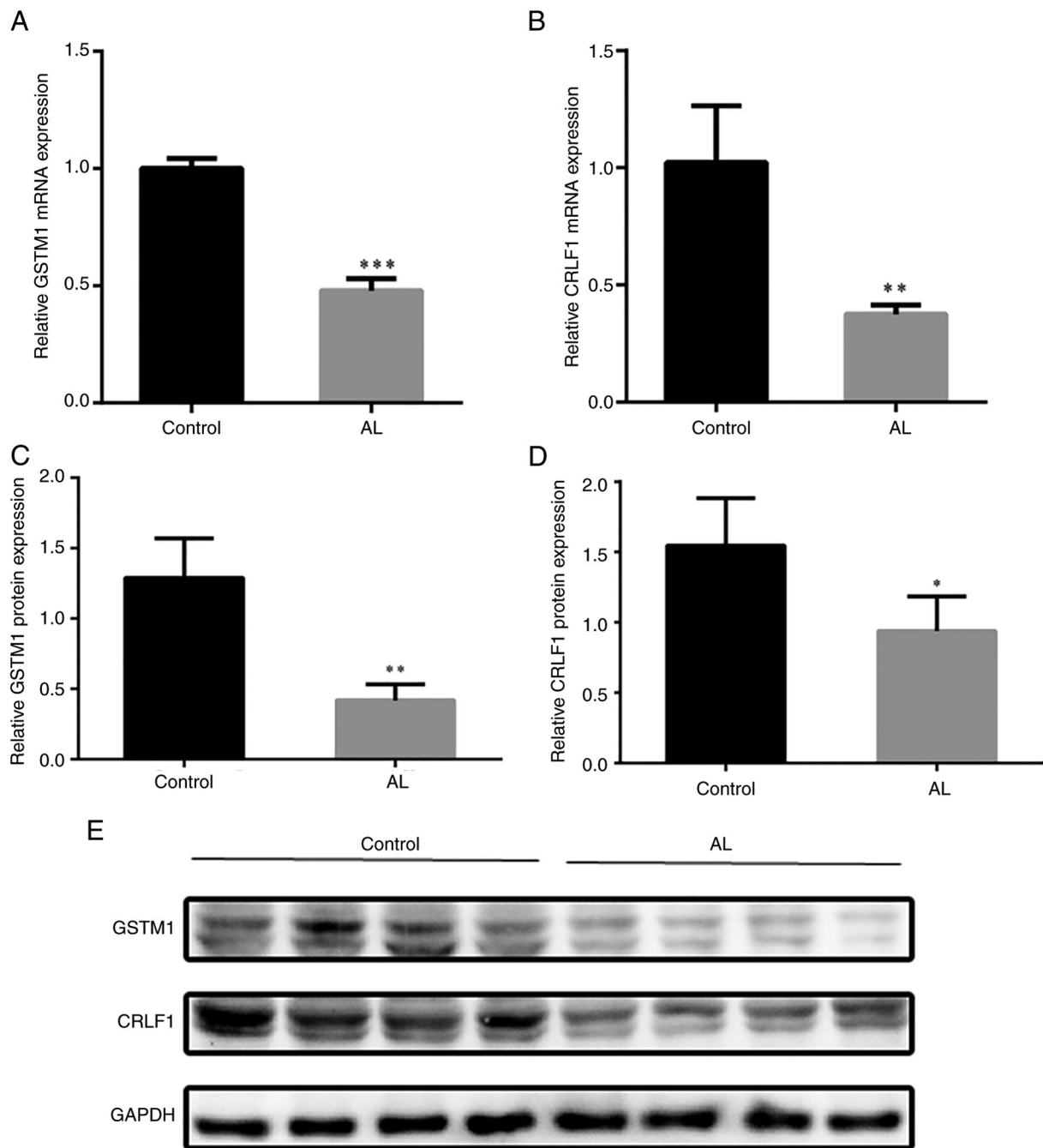


Figure 6. Verification of primary differentially expressed genes. Relative expression of (A) GSTM1 and (B) CRLF1 mRNA and (C) GSTM1 and (D) CRLF1 protein. (E) CRLF1 and GSTM1 protein immunoblotting. CRLF1, cytokine receptor-like factor-1; GSTM1, glutathione-S transferase μ 1; AL, aseptic loosening. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

the AL group and was involved in the glutathione metabolic pathway. Studies have found that glutathione accelerates osteoclast differentiation and inflammatory bone destruction, indicating that glutathione is a key molecule in the mechanisms of osteoclast and inflammatory bone destruction (44,45).

Purine metabolism serves a key role in bone metabolism and remodeling through coordination of purine receptor networks (46). Adenosine derivatives are locally released in bone by osteoblasts or osteoclasts that form bone tissue, acting directly through mechanical loading and indirectly through systemic hormones (47). Under physiological conditions, intracellular concentration of adenosine is low, while

under pathological conditions such as hypoxia, stress or inflammation, it increases (46). Locally released adenosine mediates physiological processes through its interaction with G protein-coupled receptors (46). Bone marrow cells from adenosine A1 receptors (A1Rs)-knockout mice produce fewer osteoclasts than those from wild-type mice and A1R antagonists inhibit formation of osteoclasts with reduced bone resorption capacity, indicating that adenosine serves a crucial role in bone homeostasis through its interaction with adenosine (48). N6 methyladenosine is a methylated adenosine nucleotide and its methylation promotes proliferation, differentiation and apoptosis of bone marrow mesenchymal stem

cells, osteoblasts and osteoclasts by regulating expression of alkaline phosphatase, Runx2, osterix and VEGF (49). Nitric oxide, bicarbonate, atrial, brain and C-type natriuretic peptide (CNP), guanosine, uridine and guanylate cyclase-activating protein activate guanosine, guanylate or guanylate cyclase (GC) to catalyze the conversion of guanosine triphosphate into cyclic (c)GMP and pyrophosphate (50). 8-Nitro-cGMP is a downstream molecule of nitric oxide and ROS that can promote RANKL-induced osteoclast differentiation (51). CNP activates GC-B to catalyze the synthesis of cGMP in chondrocytes and osteoblasts. Elevated cGMP stimulates long-bone growth and GC-B-dependent bone formation in mice is associated with early juvenile process, which require an increase in osteoblasts and a decrease in osteoclasts (52). These data collectively indicate that adenosine, guanine and associated enzymes are all associated with biological activity of osteoblasts and osteoclasts in bone metabolism. In the AL group, guanosine 3'-monophosphate, deoxyguanylic acid, adenosine 3'-monophosphate, guanine, L-glycine and adenosine were significantly upregulated, suggesting they may affect the activity of osteoblasts and osteoclasts and participate in occurrence and development of AL.

Arginine and proline are functional amino acids that exert anti-inflammatory and antioxidant effects in treatment of inflammation-associated diseases such as osteoarthritis (53). Proline/arginine-rich end leucine-rich repeat protein is a peptide corresponding to the N-terminal heparin-binding domain of the matrix protein proline/arginine-rich terminal leucine repeat protein, which inhibits osteoclast generation and entry into pre-fusion osteoclasts via chondroitin sulfate-dependent and membrane-associated protein 2-dependent mechanisms, decreasing nuclear factor- κ B transcription factor activity, which counteracts bone loss induced by increased osteoclast activity in various bone disease models *in vivo* (54). In bone loss, the G protein-coupled receptor Gpr54 recruits active Src and dual specificity phosphatase 18 (Dusp18) at its C-terminus, which is rich in proline/arginine. Kisspeptin-10 (Kp-10)/Gpr54 inhibits bone resorption via Dusp18-mediated Src dephosphorylation (55). In the AL group, the metabolite 1-pyrroline-2-carboxylic acid associated with the arginine and proline metabolic pathway was significantly elevated, indicating that abnormal metabolism of this metabolite may affect the arginine and proline metabolic pathway.

Due to the limitations of clinical sample collection, the present study did not obtain paired hip joint samples or pre- and postoperative tissues. Mouse calvarial osteolysis induced by titanium particles is a classic model to simulate the loosening of artificial prostheses (56). Due to the ability to test the host response in an orthotopic bone site, speed of developing osteolysis, availability of quantified images of bone loss and relatively low cost, the cranial model is the most widely used for the study of particle-induced osteolysis (57,58). Therefore, future studies should construct mouse cranial osteolysis models.

In summary, CRLF1 and GSTM1 were identified as potential biomarkers of AL based on transcriptomics and proteomics analysis of samples from AL and control subjects. The transcriptomic, proteomic and metabolomic data were integrated to describe key immune metabolic pathways associated with AL. Amino acid metabolism, including arginine and proline metabolism, and lipid metabolism, such as adenosine

and guanine and L-glycine metabolism, were involved in AL and altered metabolites may provide useful diagnostic and therapeutic biomarkers.

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Availability of data and materials

The data generated in the present study may be found in the National Center for Biotechnology Information, iproX and OMIX database under accession numbers PRJNA1160056, PXD058886 and PRJCA030476, respectively, or at the following URLs: <https://www.ncbi.nlm.nih.gov/sra/?term=SRP533942>, <https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PX058886> and ngdc.cncb.ac.cn/omix/release/OMIX007477.

Authors' contributions

YKL and JZ conceived and designed the study. YHD, XML, SQ, MEL and ZS analyzed and interpretation of data, YHD, XML, SQ and MEL wrote the manuscript. YKL and ZS edited the manuscript. XZ and ZHY analyzed data. All authors have read and approved the final manuscript. YKL and JZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was conducted according to the principles of the 1975 Declaration of Helsinki and was approved by the Medical Ethics Committee of Henan Provincial People's Hospital (Zhengzhou, China; approval no. 2022-68). Written informed consent was secured from all participants for involvement and use of their tissue samples.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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