LETTER TO THE EDITOR
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Immunological Insights into the PI3K-Akt Pathway in Osteoporosis and Periodontitis: A Proteomic and Metabolomic Approach

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Dear Editor,

Osteoporosis (OP) and periodontitis (PD) are common chronic conditions in the elderly population. Both diseases significantly impact patients' quality of life and are closely linked to systemic health issues. OP patients suffer a decrease in bone mineral density and the deterioration of bone microstructure, with an increased risk of fractures.[1] PDis an inflammatory disease caused by bacterial infection, marked by symptoms such as gum bleeding and alveolar bone loss. Research suggests these 2 diseases may share common pathological mechanisms, particularly in immune-inflammatory responses and bone metabolism.[2] The PI3K-Akt pathway is a crucial regulator in various physiological and pathological processes.

Most studies focus on local inflammatory responses or specific molecular markers, such as bone metabolism markers and inflammatory cytokines. Research has shown that bone metabolism-related markers, such as alkaline phosphatase and osteocalcin, exhibit significant expression changes in OP patients. In patients with PD, a substantial rise in pro-inflammatory cytokines, including interleukin (IL)-1β and tumor necrosis factor-alpha (TNF-α), was observed compared to other groups. The significance of the PI3K-Akt pathway in regulating bone metabolism is well acknowledged. It is critical in regulating bone resorption and formation and modulating inflammatory and immune responses.[3] The PI3K-Akt pathway regulates the growth and differentiation of osteoblasts and osteoclasts by activating downstream targets such as mTOR and GSK3β, thereby maintaining the dynamic balance of bone tissue.[4] The improper activation of the PI3K-Akt pathway could potentially contribute to the progression of OP.[5]

The main objective of this paper is to investigate the common pathogenesis of OP and periodontitis by integrating proteomics and metabolomics analyses, with special emphasis on the role of the PI3K-Akt pathway. First, we will analyze proteomic data from OP, PD, and OP-PD (comorbid OP and PD) samples to identify differentially expressed proteins associated with these diseases. Subsequently, metabolomic analysis will identify metabolic changes related to the PI3K-Akt pathway. In addition, network pharmacology approaches will be employed to explore potential therapeutic strategies. Through this research, we aim to provide novel insights into the molecular mechanisms and therapeutic targets, offering new directions for treating OP and PD.

In this paper, we first used the proteomics analysis method. Serum samples were collected from 4 groups: healthy controls (C, n=6), PD (n=6), OP (n=6), and PD+OP (n=3). The inclusion criteria for each group were as follows:

For the healthy control group: 1) No history of periodontitis, OP, or other systemic diseases affecting

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bone metabolism. 2) Not taking medications known to influence bone health.

For the PD group: 1) Diagnosed with moderate to severe chronic periodontitis. 2) Clinical parameters: probing depth ≥4 mm, clinical attachment loss≥3 mm, and visible alveolar bone loss on X-ray.

For the OP group: 1) Diagnosed with primary OP. 2) Bone mineral density T-score ≤−2.5 at the lumbar spine or hip.

For the combined PD+OP group: 1) Met diagnostic criteria for both PD and OP.

Exclusion criteria for all groups: 1) History of cancer, severe liver or kidney disease, or autoimmune diseases. 2) Use of bisphosphonates, hormone replacement therapy, or other bone metabolism-affecting medications within the past year. 3) Presence of infections or acute inflammatory conditions.

Samples were stored at −80°C. Total proteins were isolated using RIPA buffer, and protein concentrations were determined using the bicinchoninic acid (BCA) kit. Proteins were digested with trypsin at 37°C for 16 hours. The resulting peptides were analyzed by mass spectrometry after vacuum centrifugation to remove urea. Peptides were separated on an ultra-performance liquid chromatography (UPLC) system and analyzed using a Q Exactive HF-X mass spectrometer. Data were processed with MaxQuant software, and proteins were identified against the UniProt database using tandem mass tag (TMT) labeling for quantification. Proteins with $p<0.05$ and fold change>1.5 were considered significantly different.

Regarding the metabolomics analysis, untargeted metabolomics was performed using an ultra-high performance liquid chromatography coupled with a quadrupole orbitrap mass spectrometry (UHPLC-Q Exactive-MS) system to analyze metabolic alterations in serum samples from different groups. For metabolite extraction, 200 μL of serum was mixed with extraction solvent containing an internal standard (L-2-chlorophenylalanine, 0.3 mg/mL in acetonitrile). Samples were homogenized, sonicated, and incubated to precipitate proteins. After centrifugation, supernatants were transferred for analysis. Metabolite separation was performed on a Vanquish Horizon UHPLC system with a BEH C18 column. Chromatographic separation was conducted at 40°C with a 0.4 mL/min flow rate using a gradient elution program. Detection was carried out using a Q Exactive mass spectrometer, alternating between positive and negative ion modes. Mass spectrometry parameters were optimized, and quality control (QC) samples were analyzed periodically to ensure reliability and reproducibility. Raw data were processed using Xcalibur and Compound Discoverer software, and data analysis was conducted using platforms such as MetaboAnalyst. Metabolites were identified by matching data against public databases (HMDB, KEGG, METLIN). Multivariate statistical methods, such as principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), were used to evaluate metabolic variations, and significant metabolites were selected based on *t* tests and analysis of variance (ANOVA, $p<0.05$) with Benjamini-Hochberg correction. Functional annotation and pathway analysis were conducted using databases (KEGG, Reactome) and network pharmacology approaches. Metabolite-pathway networks were constructed using Cytoscape software.

Gene set enrichment analysis was performed using GSEA software (v4.2.3) on gene expression data from four groups: healthy controls (C), PD(PD), OP (OP), and comorbid PD+OP. Differentially expressed gene sets were generated, and raw data were log2 transformed and normalized before input into GSEA. Gene sets were derived from MSigDB (v7.4). Wilcoxon rank-sum test identified significant genes, and the Enrichment Score (ES) was calculated. Significance was assessed with false discovery rate (FDR) <0.25 and $p<0.05$.

We also used Weighted Gene Co-expression Network Analysis (WGCNA) to construct a protein co-expression network and identify regulatory factors related to OP and PD. Analysis was conducted in R (v4.0.3) using the WGCNA package (v1.73). A soft-thresholding power was selected to ensure scale-free topology. Hierarchical clustering identified gene modules, which were visualized with a heatmap created using the heatmap package (v1.0.12).

Total RNA was isolated from patient serum samples using TRIzol reagent and reverse-transcribed into cDNA with the quantitative polymerase chain reaction (qPCR) RT kit. PCR was performed using SYBR Green PCR Master Mix. Target genes included APOL1, SRGN, BGH3, PIP, LYAM1, VWF, and PCOC1. Relative expression levels to GAPDH were calculated using the $2^{-\Delta\Delta Ct}$ method. Experiments were conducted in triplicate.

Total protein was extracted from serum samples using RIPA lysis buffer and measured with a BCA Protein Assay Kit. 10% sodium dodecyl sulfate-

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polyacrylamide gel electrophoresis (SDS-PAGE) separated protein samples (20 μg), transferred to a polyvinylidene fluoride (PVDF) membrane, and blocked with 5% non-fat milk. The membrane was incubated with primary antibodies against CD44, IGKV2, PF4, and GP5 (all 1:1000, Abcam) overnight at 4°C, followed by secondary antibody incubation for 2 hours at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL) Plus reagent and quantified with ImageJ.

Statistical analyses were performed using R software (v4.0.0 or above). Group differences were analyzed using one-way ANOVA, with Benjamini-Hochberg correction for multiple comparisons. $p<0.05$ was considered significant.

Proteome variations were identified in OP and PD pathogenesis. Comparing protein expression between disease groups and controls, we found differentially expressed proteins in each group (Supplementary Figure 1A). PCA showed significant separation between disease and control groups, with overlap between OP, PD, and OPPD (OP and PD comorbidity) samples (Supplementary Figure 1B). Unsupervised clustering and expression pattern analysis revealed distinct protein expression profiles, suggesting OP may contribute to PD development (Supplementary Figure 1C–D).

The PI3K-Akt pathway emerged as a key player in the pathogenesis of both OP and PD, with consistent findings across proteomics and metabolomics analyses. Proteomics data revealed significant alterations in proteins associated with the PI3K-Akt pathway, which were validated by RT-PCR and Western blotting (Supplementary Figures 2C, 2D, 3D, and 3E). Metabolomics analysis complemented these findings by showing corresponding changes in metabolites linked to this pathway, indicating its central role in disease progression (Supplementary Figures 4E and 5C). The convergence of evidence from both omics approaches underscores the importance of the PI3K-Akt pathway in the shared pathogenesis of OP and PD.

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Key proteins and metabolites were identified through integrated network analyses. ANOVA identified five core differential proteins (CD44, IGKV2, DEFA, PF4/CXCL4, and GP5/CD42d) significantly different across groups (Supplementary Figure 3B). Self-organizing neural network clustering and heatmap analysis highlighted distinct modules and upregulation of these proteins in disease groups, with the highest expression in OPPD (Supplementary Figures 3C–D). Upset analysis further revealed 26 common differential metabolites across groups, with pathway analysis highlighting six significant pathways, including butanoate and tryptophan metabolism (Supplementary Figures 5A–B). Pearson correlation analysis showed a close correlation between protein expression in biosynthetic pathways and metabolite levels (Supplementary Figures 5D–E).

A co-expression regulatory network was constructed using WGCNA, identifying potential regulatory factors in the pathogenesis of OP and PD. An appropriate soft-thresholding power ensured a scale-free topology model (Supplementary Figure 6A). Multiple modules were identified, with strong associations between specific modules and disease states (Supplementary Figures 6B–C). A weighted network of key proteins from significant modules revealed critical regulatory roles (Supplementary Figure 6D). A heatmap showed a high expression of these factors in disease groups (Supplementary Figure 6E).

Network pharmacology analysis identified active components of Angelica sinensis and their potential targets in treating OP and PD. Active components included glycosides, flavonoids, and organic acids. Through network pharmacology analysis, we also identified the potential targets of these active components. KEGG enrichment analysis highlighted involvement in critical signaling pathways, including the PI3K-Akt pathway (Supplementary Figure 7A). A network of active components and potential targets was constructed (Supplementary Figure 7B). Molecular docking simulations showed a high affinity of Glycitein for CD44 and Pinosylvin for AKT1, with detailed binding modes illustrated (Supplementary Figure 7C–F).

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This study used proteomics and metabolomics to find that OP and PD share molecular traits, with the PI3K-Akt pathway being key in both. The proteomic analysis found key proteins in this pathway that changed in both diseases. Metabolomic analysis showed significant differences in metabolite expression linked to these proteins. PCA and PLS-DA showed metabolic similarities between OP, PD, and OP-PD groups, differing from controls. This suggests OP may raise PD risk via the PI3K-Akt pathway.

The PI3K/Akt pathway, involved in cell processes like proliferation and adhesion, affects osteoblast and osteoclast functions, influencing OP. In postmenopausal OP, its dysregulation boosts bone resorption. PD, a chronic inflammatory disease, activates immune pathways like PI3K/Akt, worsening inflammation. Akt, a key molecule in PI3K signaling, regulates immune responses by activating transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).

Angelica sinensis, a traditional herb, may treat OP and PD by modulating immune responses and bone metabolism. Its active compounds may regulate bone density via the PI3K/Akt pathway, suppressing bone resorption and promoting formation. They may also reduce inflammation in PD by modulating immune pathways. Molecular docking showed strong binding affinities of components like Glycitein and Pinosylvin to key proteins.

However, it is important to acknowledge the limitations of our study. The experiments were primarily conducted in vitro, which limits the generalizability of our findings to in vivo conditions. The complex interplay of cells and the immune system in a living organism cannot be fully replicated in vitro, suggesting that our results should be interpreted with caution. Additionally, our study lacks clinical follow-up or validation, which means the translational value of our findings remains speculative. Future research should validate these findings using in vivo models. Animal studies could provide a more comprehensive understanding of the PI3K-Akt pathway's role in the pathogenesis of OP and PD. For instance, experiments using knockout mice or pharmacological modulation of the PI3K-Akt pathway could clarify its in vivo function. Additionally, longitudinal studies in animal models could help establish the temporal relationship between PI3K-Akt pathway activation and disease progression. These in vivo experiments would also allow for the examination of the pathway's effects on bone and gum tissue in a more natural disease context, potentially revealing additional therapeutic targets and mechanisms that were not evident in our in vitro analysis. Furthermore, clinical studies involving patients with OP and PD are needed to assess the efficacy and safety of potential therapeutic interventions targeting the PI3K-Akt pathway. Such studies would provide crucial insights into the clinical relevance of our findings and pave the way for the development of novel treatments for these conditions.

STATEMENT OF ETHICS

This study was approved by the Ethics Committee of Gansu Provincial Hospital. Signed written informed consents were obtained from the patients and/or guardians. This study was conducted in accordance with the Declaration of Helsinki.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Not applicable.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

Not applicable.

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