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ORIGINAL RESEARCH

Single-Cell and Transcriptome Analysis of Periodontitis: Molecular Subtypes and Biomarkers Linked to Mitochondrial Dysfunction and Immunity

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Background: Periodontitis represents an inflammatory disease with multiple contributing factors, affecting both oral and systemic health. The mechanisms linking mitochondrial dysfunction to immune responses in periodontitis remain unclear, limiting the development of individualized diagnostic and therapeutic approaches.

Objective: This study aims to elucidate the roles of mitochondrial dysfunction and immune responses in the pathogenesis of periodontitis, identify distinct molecular subtypes, and discover robust diagnostic biomarkers to support precision medicine approaches.

Methods: Single-cell RNA sequencing and transcriptome data from periodontitis patients were analyzed to identify gene signatures linked to macrophages and mitochondria. Consensus clustering was applied to classify molecular subtypes. Potential biomarkers were identified using five machine learning algorithms and validated in clinical samples through qPCR and IHC.

Results: Four molecular subtypes were identified: quiescent, macrophage-dominant, mitochondria-dominant, and mixed, each exhibiting unique gene expression patterns. From 13 potential biomarkers, eight were shortlisted using machine learning, and five (BNIP3, FAHD1, UNG, CBR3, and SLC25A43) were validated in clinical samples. Among them, BNIP3, FAHD1, and UNG were significantly downregulated ($p < 0.05$).

Conclusion: This study identifies novel molecular subtypes and biomarkers that elucidate the interplay between immune responses and mitochondrial dysfunction in periodontitis. These findings provide insights into the disease's heterogeneity and lay the foundation for developing non-invasive diagnostic tools and personalized therapeutic strategies.

Keywords: periodontitis, single-cell RNA sequencing, mitochondrial dysfunction, immune microenvironment, molecular subtypes, machine learning

Introduction

Periodontitis is a multifactorial inflammatory disease that compromises the supporting structures of the teeth, leading to significant oral and systemic health complications. Traditionally classified as a bacterial-induced condition, emerging evidence underscores the crucial roles of host immune responses and cellular metabolic dysregulation in its pathogenesis.[1–4] Among these, mitochondrial dysfunction has garnered increasing attention for its crucial role in various inflammatory diseases, including periodontitis.[5]

Recent advances in single-cell RNA sequencing (scRNA-seq) have significantly enhanced our understanding of cellular heterogeneity and molecular pathways in complex diseases, including periodontitis.[6] This technology facilitates the identification of distinct cellular populations and their functional roles within the periodontal microenvironment. However, most studies have concentrated on limited data types or specific cell populations, which fail to capture the molecular complexity

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and heterogeneity of periodontitis.[7–12] These gaps impede the identification of molecular subtypes, which may account for variations in disease progression and treatment outcomes.

Mitochondria are essential for energy production, metabolism, and immune regulation.[13,14] Mitochondrial dysfunction contributes to periodontitis through multiple mechanisms:[15] producing excessive reactive oxygen species (ROS) leading to oxidative stress, impairing autophagy, releasing mitochondrial DNA that triggers inflammatory cytokine production, inducing mitochondria-mediated apoptosis that affects periodontal tissue cells,[16] and disrupting mitochondrial dynamics which impacts cellular function and survival.[17,18] These interconnected mechanisms create a vicious cycle of inflammation and tissue destruction, promoting disease progression.[19]

This study combines macrophage-related genes from scRNA-seq with mitochondrial-related genes from transcriptome data to investigate the relationship between mitochondrial dysfunction and immune responses in periodontitis. Using multi-omics approaches and machine learning, it aims to classify molecular subtypes and uncover diagnostic biomarkers. While machine learning has proven effective in biomarker discovery across various medical fields, its potential remains underexplored in periodontitis research.[20]

Current treatments for periodontitis, such as quadrant-wise or full-mouth subgingival instrumentation, effectively manage symptoms but do not address the underlying molecular mechanisms.[21] Recent advances in understanding the molecular heterogeneity of periodontitis, including the identification of subtypes and biomarkers, suggest that integrating molecular diagnostics with existing therapies could improve treatment precision and efficacy.[22] Periodontitis poses significant diagnostic challenges, as current clinical methods are inadequate for early detection and predicting disease progression. Molecular biomarkers, including inflammatory mediators, immune-regulatory molecules, and mitochondrial-related genes, offer promising solutions for enhancing diagnostic accuracy and distinguishing disease subtypes. Furthermore, identifying robust biomarkers can facilitate personalized treatment strategies by linking specific molecular signatures to targeted therapeutic approaches. Despite these advances, there remains a critical need to integrate multi-omics data to identify reliable diagnostic markers tailored to the molecular heterogeneity of periodontitis. To address this gap, this study aims to integrate single-cell RNA sequencing and transcriptome data to identify molecular subtypes and diagnostic biomarkers of periodontitis, with the ultimate goal of establishing a foundation for precision medicine approaches that combine molecular insights with conventional treatments to enhance disease management.

Materials and Methods

Data Acquisition and Processing

Single-cell RNA sequencing (scRNA-seq) data of oral mucosal tissue from eight periodontitis patients (GSE164241) and whole transcriptome datasets (GSE10334, GSE16134, GSE23586, and GSE106090) were obtained from the Gene Expression Omnibus (GEO) database.[23–26] Datasets were included based on the following criteria: original scRNA-seq or whole transcriptome data derived from periodontal tissues, clear definition of periodontitis and control groups with a minimum sample size of three per group, comprehensive clinical information and experimental protocols, and availability in peer-reviewed journals with public access. Exclusion criteria included duplicate datasets, insufficient experimental details, unclear disease classification, low-quality sequencing data, non-standard protocols, or focus on other oral diseases without periodontitis involvement.

To ensure robustness, multiple independent datasets were integrated, standardized processing protocols were implemented, and batch effects were corrected using the "Combat" algorithm from the "sva" R package. Cross-validation was performed using diverse analytical approaches, and data quality was assessed through stringent quality control measures. Additionally, mitochondria-related genes were curated from the Human MitoCarta3.0 database (broadinstitute.org). These measures ensured the reliability and reproducibility of data for downstream analyses.

Quality Control and Data Preprocessing

The 'Seurat' R package was used for quality control and analysis (Supplementary Figures 1 and 2). Cells were filtered based on the following criteria: gene count between 500 and 4000 (minGene = 500; maxGene = 4000), UMI count below 15,000 (maxUMI = 15,000), mitochondrial gene percentage below 5% (pctMT = 5), and hemoglobin gene percentage

below 1% (pctHB = 1). These criteria were established to exclude low-quality cells or doublets. A low gene count or high mitochondrial content often indicates stressed or dying cells, while high hemoglobin content may suggest contamination from red blood cells.

Data Integration and Dimensionality Reduction

The "RunHarmony" function was employed to integrate samples and correct for batch effects while preserving biological variability. Principal Component Analysis (PCA) was conducted on the top 3000 highly variable genes using the "RunPCA" function. The first 10 principal components (PCs) were selected for further analysis based on the elbow plot and the variance explained by each component.

Clustering and Cell Type Annotation

The 'clustertree' R package was utilized to determine the optimal resolution (0.3) for clustering. The "FindClusters" function identified cell clusters, which were visualized using the "RunUMAP" function. Cell types were manually annotated based on known cell-specific lineage gene expression.

Differential Expression and Functional Enrichment Analysis

Differentially expressed genes in each cell cluster were identified using the "FindAllMarkers" function. Functional enrichment analysis was performed with the SCP package, utilizing GO-BP, KEGG, and Reactome databases for pathway analysis. Word cloud analyses for GO Molecular Function (GO-MF) and GO Cellular Component (GO-CC) terms were generated using the "RunEnrichment" function. However, macrophage-related differentially expressed genes were identified through whole transcriptome sequencing data, not from single-cell RNA sequencing analysis. Following manual annotation, the "FindAllMarkers" function was used only for cell cluster characterization in the single-cell analysis.

Identification of Molecular Subtypes

Macrophage and mitochondria-related differentially expressed genes were identified using the Wilcoxon test based on whole transcriptome sequencing data. To define molecular subtypes of periodontitis, the ConsensusClusterPlus package (parameters: reps = 100, pItem = 0.8, pFeature = 1) was employed for consensus clustering of the differentially expressed macrophage and mitochondrial genes. Ward.D2 and Euclidean distances were selected as the clustering algorithm and distance metric, respectively, with k = 4. Samples were assigned to four subgroups based on the median expression levels of co-expressed macrophage and mitochondrial genes: Quiescent (macrophage genes < 0, mitochondrial genes < 0), Macrophage-dominant (macrophage genes > 0, mitochondrial genes < 0), Mitochondria-dominant (macrophage genes < 0, mitochondrial genes > 0), and Mixed (macrophage genes > 0, mitochondrial genes > 0).

Characterization of Molecular Subtypes

The immune characteristics of the identified molecular subtypes were analyzed to assess their biological significance. Differences in HLA gene expression between subgroups were evaluated using the Kruskal–Wallis test. The periodontal microenvironment was characterized using three computational methods: MCPcounter, quantiseq, and ssGSEA. For multivariate comparisons, the Kruskal–Wallis test was used, while pairwise comparisons were conducted using the Wilcoxon test.

Identification of Key Diagnostic Genes

Five machine learning algorithms were employed to identify robust biomarkers for periodontitis: Least Absolute Shrinkage and Selection Operator (LASSO) regression, Support Vector Machine Recursive Feature Elimination (SVM-RFE), Random Forest and Boruta (RFB), and Extreme Gradient Boosting (XGBoost). LASSO regression was used for its ability to introduce regularization and prevent overfitting, making it suitable for high-dimensional datasets. Random Forest aggregates multiple decision trees to capture complex nonlinear interactions, thus identifying subtle patterns in the data. These algorithms were implemented using the R packages "glmnet", "rms", "sigFeature", "e1071", "randomForest", "Boruta", and "XGBoost".[27–30] Genes selected by all five methods were considered key diagnostic

markers. The area under the Receiver Operating Characteristic (ROC) curve (AUC) was calculated to evaluate the diagnostic performance of these markers using the 'pROC' R package.

Human Sample Collection and Specimen Histology

Gingival tissue specimens were collected from two distinct groups. The control group comprised 8 healthy individuals undergoing crown lengthening procedures, while the periodontitis (PD) group included 8 patients diagnosed with severe PD requiring periodontal flap surgery. All participants provided informed consent prior to tissue collection. Demographic information, including age and gender, along with specific exclusion criteria for all eight participants, is provided in Table 1. Exclusion criteria included systemic diseases such as diabetes and ongoing use of anti-inflammatory drugs or antibiotics to minimize potential confounding factors.

Quantitative PCR (qPCR)

Total RNA was extracted from gingival tissue samples using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from 1 μg of total RNA using a reverse transcription kit (Thermo Fisher). qPCR was performed under the following conditions: 95°C for 30 seconds, followed by 45 cycles of 95°C for 5 seconds and 60°C for 34 seconds. Relative gene expression was calculated using the ΔΔCt method. Primer sequences are shown in Table 2.

Immunohistochemistry (IHC)

Immunohistochemistry was performed on paraffin-embedded gingival tissue sections (4 μm). After deparaffinization and rehydration, antigen retrieval was performed using sodium citrate buffer (pH 6.0) in a microwave. Sections were blocked with 3% hydrogen peroxide and incubated overnight at 4°C with primary antibodies for BNIP3, FAHD1, UNG, SLC25A43, and CBR3. After washing, sections were treated with secondary antibodies, followed by DAB staining and hematoxylin counterstaining. Staining was evaluated under a light microscope, and ImageJ software was used for semi-quantitative analysis of staining intensity.

Statistical Analysis

All statistical analyses were conducted using R software and GraphPad Prism (version 10.0, GraphPad Software, San Diego, CA). P-values < 0.05 were considered statistically significant. Multiple testing corrections were applied using the Benjamini-Hochberg method to control the false discovery rate where necessary.

Table 1 The Detail of Clinical Information

	Control	Periodontitis
	(patient, n=8)	(patient, n=8)
Age (Mean)	32	38
Gender (M/F)	3/5	5/3
GI (Mean)	0	3.2
Explore clinical depth (Mean)	2.5	6.5
System disease (Yes/No)	0/8	0/8
Smoking history (Yes/No)	0/8	0/8
Medication history (Yes/No)	0/8	0/8
Alveolar bone resorption (Yes/No)	0/4	8/0
BOP+ (Yes/No)	2/6	8/0

Table 2 Experimentally Relevant Primers

Gene Name	Forward Primer	Reverse Primer
h-SLC25A43F	TCTCTCCCACAGAACTTTGCTAAT	CTGCTCCTGAGAAATGGACATCT
h-FAHD1	TTATCTTGACTGGGACGCCAAAG	CCCTTCATTCCTGTAGTCTCACA
h-BNIP3	GAACACGAGCGTCATGAAGAAAG	ACCAAGTCAGACTCCAGTTCTTC
h-CBR3	GCTCAACGTACTGGTCAACAAC	CAGTAACTCGTTGCACATGTTTCT
h-UNG	AGCTAATGGGATTTGTTGCAGAAG	GGAACAGGCCTTTGAACACTAAA
GAPDH	ATTCCATGGCACCGTCAAGG	TGGACTCCACGACGTACTCA

Results

Single-Cell Transcriptomics Reveals Cellular Heterogeneity in Periodontal Tissue

To characterize the cellular composition of periodontal tissue in periodontitis, we analyzed publicly available scRNA-seq data from the GEO database. After quality control and filtering, unsupervised clustering analysis at a resolution of 0.3 identified 11 distinct cell clusters (numbered 0–10), as visualized by Uniform Manifold Approximation and Projection (UMAP) (Figure 1A). By using the elbow plot and analyzing cluster stability at various resolutions, we determined that a resolution of 0.3 provided the optimal separation of cell populations. This resolution preserved sufficient heterogeneity while avoiding over-clustering.

The relative proportions of these cell clusters varied across different samples, as illustrated in the stacked bar plot (Figure 1b). This variability suggests potential inter-individual differences or disease-related alterations in cellular composition within the periodontal microenvironment.

These cell clusters were characterized by examining the expression of known cell type-specific markers. A heatmap of marker gene expression across clusters revealed distinct transcriptional signatures for each population (Figure 1c). The color gradient from purple to yellow indicates low to high expression levels, respectively.

Further annotation of cell types was achieved through a dot plot representation of key marker genes (Figure 1d). The size of each dot correlates with the percentage of cells expressing the gene, while the color intensity indicates the average expression level. Based on this comprehensive analysis, we identified several major cell types within the periodontal tissue, including T cells (CD3D+, CD3E+, CD3G+), B cells (MS4A1+, CD79A+), plasma cells (SDC1+, MZB1+), myeloid cells (CD68+, LYZ+, FCER1A+, CD1C+), neutrophils (FCGR3B+, CXCR2+), endothelial cells (PECAM1+, VWF+), fibroblasts (COL1A1+, DCN+), and epithelial cells (KRT5+, KRT14+).

Further annotation based on specific marker genes revealed that these 11 clusters represented 12 distinct cell types, as some clusters contained closely related but functionally distinct cell populations.After analyzing the cellular composition of periodontal tissue, we explored the relationship between gene expression and biological functions in these clusters. A circular gene-function association plot (Figure 1E) was generated to visualize the key genes and their related pathways in periodontitis.

Functional Enrichment Analysis Reveals Distinct Biological Characteristics of Cell Clusters

After establishing the cellular composition of periodontal tissue, the biological functions of the identified cell clusters were further explored through functional enrichment analysis using multiple databases, including GO-BP, KEGG, and Reactome. The FeatureHeatmap function in the SCP package was employed for this analysis, adopting a strategy of mutual verification across these databases.

The resulting heatmap revealed distinct functional profiles for each cell cluster (Figure 2A). Cluster 0 exhibited strong enrichment in immune response-related processes, with high expression of key immune-related genes such as LYZ, CD68, and FCGR3A, confirming its identity as a macrophage population. Additionally, genes like CXCL8 and IL1B

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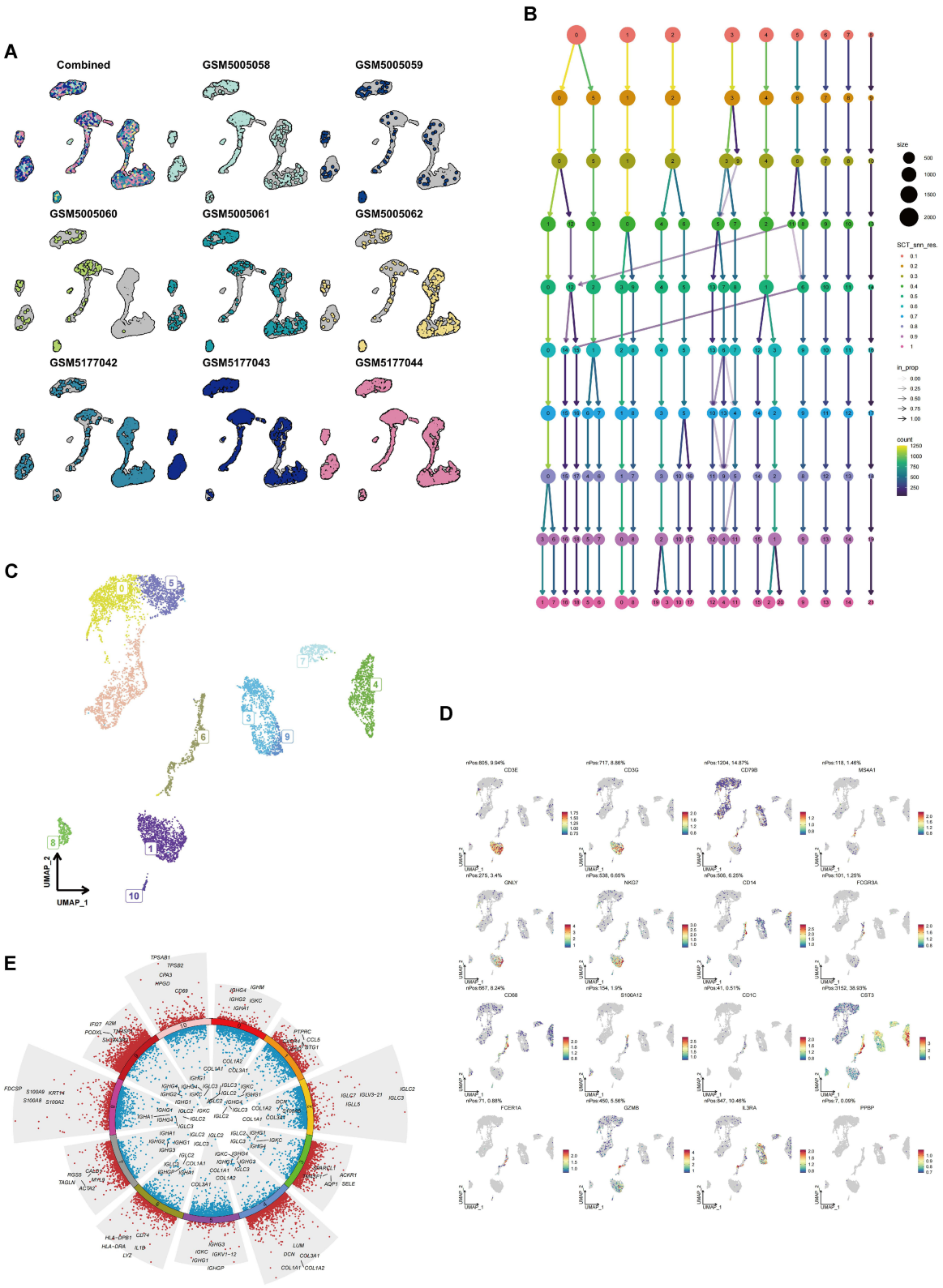


Figure 1 Cellular heterogeneity in periodontal tissue. (**A**) UMAP plot of 12 identified cell clusters. (**B**) Hierarchical tree diagram showing cluster proportions across samples. (**C**) UMAP plot displaying cell type distribution. (**D**) Dot plot of marker gene expression across clusters. (**E**) Circular plot showing key genes and functions.

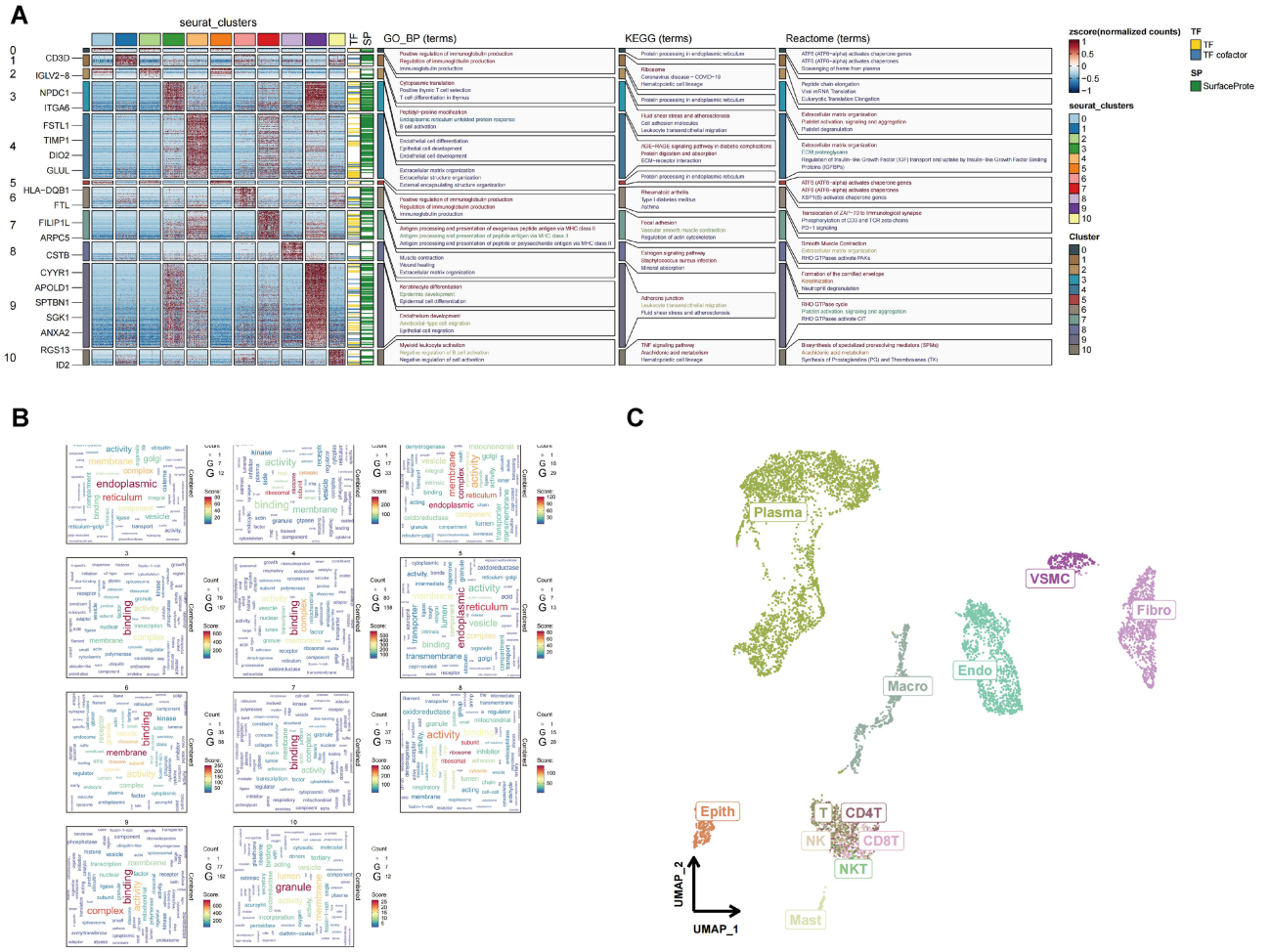
were highly expressed, suggesting that this cluster plays a critical role in pro-inflammatory responses during periodontitis.

Cluster 1 showed high enrichment in extracellular matrix organization, with genes such as COL1A1, DCN, and COL3A1 highly expressed, indicating that this cluster is composed of fibroblasts involved in collagen synthesis and tissue structural integrity. Similarly, cluster 3 was predominantly associated with cell adhesion and migration processes, with high expression of ITGA1, VIM, and FN1, indicating a role in tissue remodeling and cell movement.

The RunEnrichment function was further utilized to generate word cloud analyses for GO Molecular Function (GO-MF) and GO Cellular Component (GO-CC) terms for each cell cluster (Figure 2B). These word clouds provided a visual representation of key biological terms associated with each subgroup. For instance, cluster 0's word cloud was dominated by terms such as "cytokine activity" and "chemokine activity", corroborating its immune-related functions identified in the heatmap analysis. Cluster 5 showed terms like "angiogenesis" and "cell adhesion", supported by the expression of genes such as VEGFA and ITGB1, indicating a role in vascular development and tissue repair.

Unsupervised clustering of the transcriptomic data reaffirmed the 11 distinct cell clusters identified earlier, as visualized in the UMAP plot (Figure 2C). Each cluster, represented by a unique color, showed clear separation from others, further supporting their distinct transcriptional profiles.

Following manual annotation of cell types based on known markers, the FindAllMarkers function was used to identify cluster-specific genes. The expression patterns of these marker genes across different clusters are displayed in Figure 2D and E. Notably, cluster 0 exhibited high expression of LYZ, CD68, and FCGR3A, confirming its identity as a



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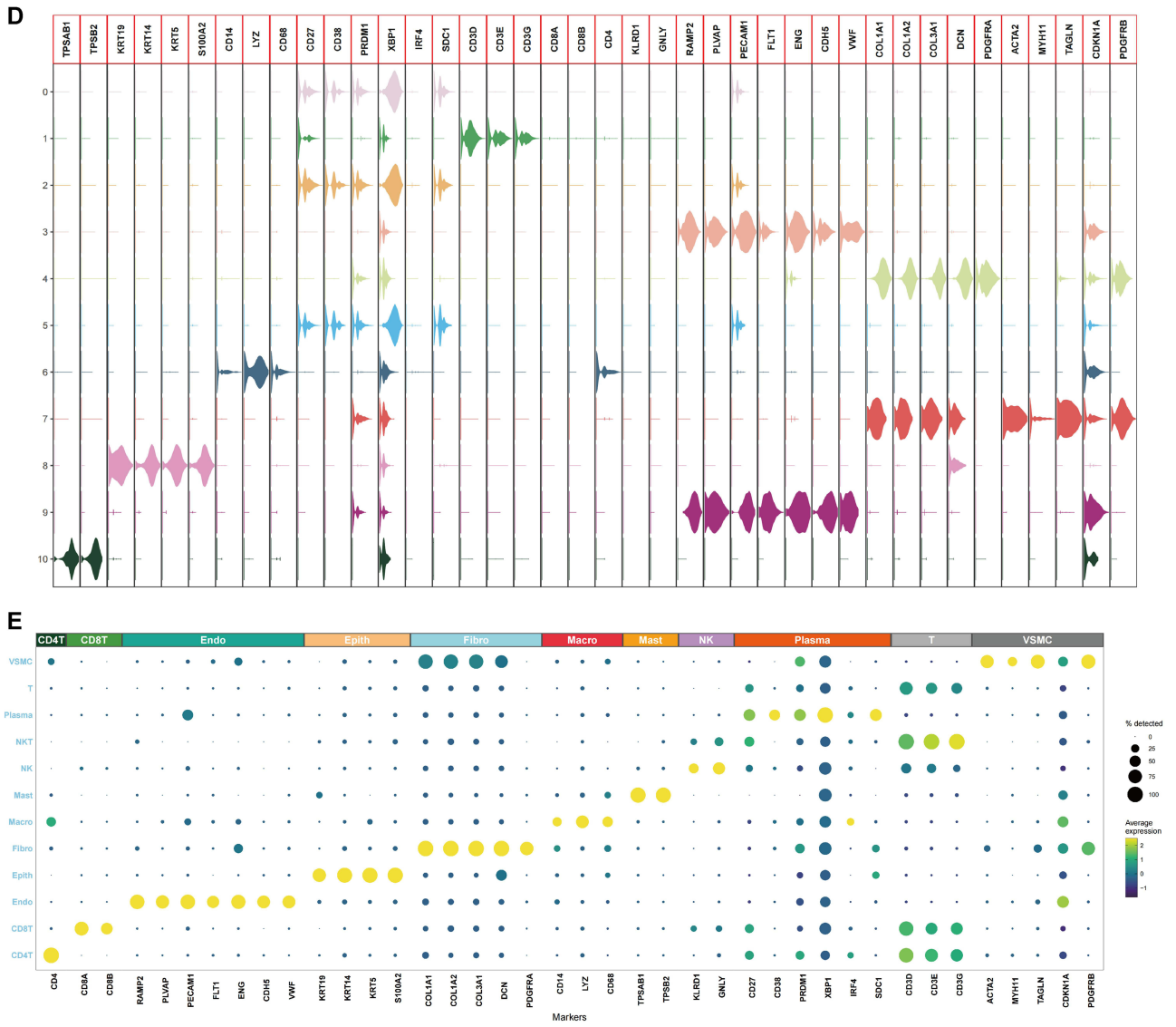

Figure 2 Functional enrichment and gene expression in cell clusters. (**A**) Heatmap showing functional enrichment (GO-BP, KEGG, Reactome) for each cluster, with color indicating enrichment level. (**B**) Word clouds of GO Molecular Function (GO-MF) and Cellular Component (GO-CC) terms, with font size representing significance. (**C**) UMAP plot showing 12 distinct cell clusters based on transcriptomic data. (**D**) Violin plots displaying marker gene expression across clusters. (**E**) Dot plot showing marker gene expression, with dot size indicating the percentage of expressing cells and color reflecting expression level.

macrophage population involved in immune responses. Other clusters, such as cluster 1 with COL1A1 and DCN (fibroblasts), and cluster 5 with VEGFA and ITGA1 (endothelial cells), displayed unique gene signatures that aligned with their annotated cell types, providing additional validation of the cell type classifications.

Identification and Characterization of Molecular Subtypes in Periodontitis

RNA-seq data from periodontitis patients were analyzed to identify gene expression patterns. Specifically, 22 macrophage-related genes and 14 mitochondrial-related genes associated with periodontitis were selected from the whole transcriptome analysis (Supplementary Table 1). To explore potential interactions between these two pathways, the co-expression patterns of macrophage and mitochondrial genes were calculated across all periodontitis samples. This analysis aimed to assess how the combined expression of these gene sets contributes to the molecular heterogeneity of the disease.

The co-expression analysis involved calculating the median expression levels of both macrophage and mitochondrial gene sets for each sample. Consensus clustering was then applied to classify the samples based on the expression of these two gene sets. The Ward.D2 clustering algorithm and Euclidean distance metric were used, with the optimal number of clusters determined as k = 4 after performing 100 iterations of consensus clustering analysis (Figure 3A and B). This process resulted in the identification of four distinct molecular subtypes: Quiescent, Macrophage-dominant, Mitochondria-dominant, and Mixed, based on the balance of macrophage and mitochondrial gene expression in each group.

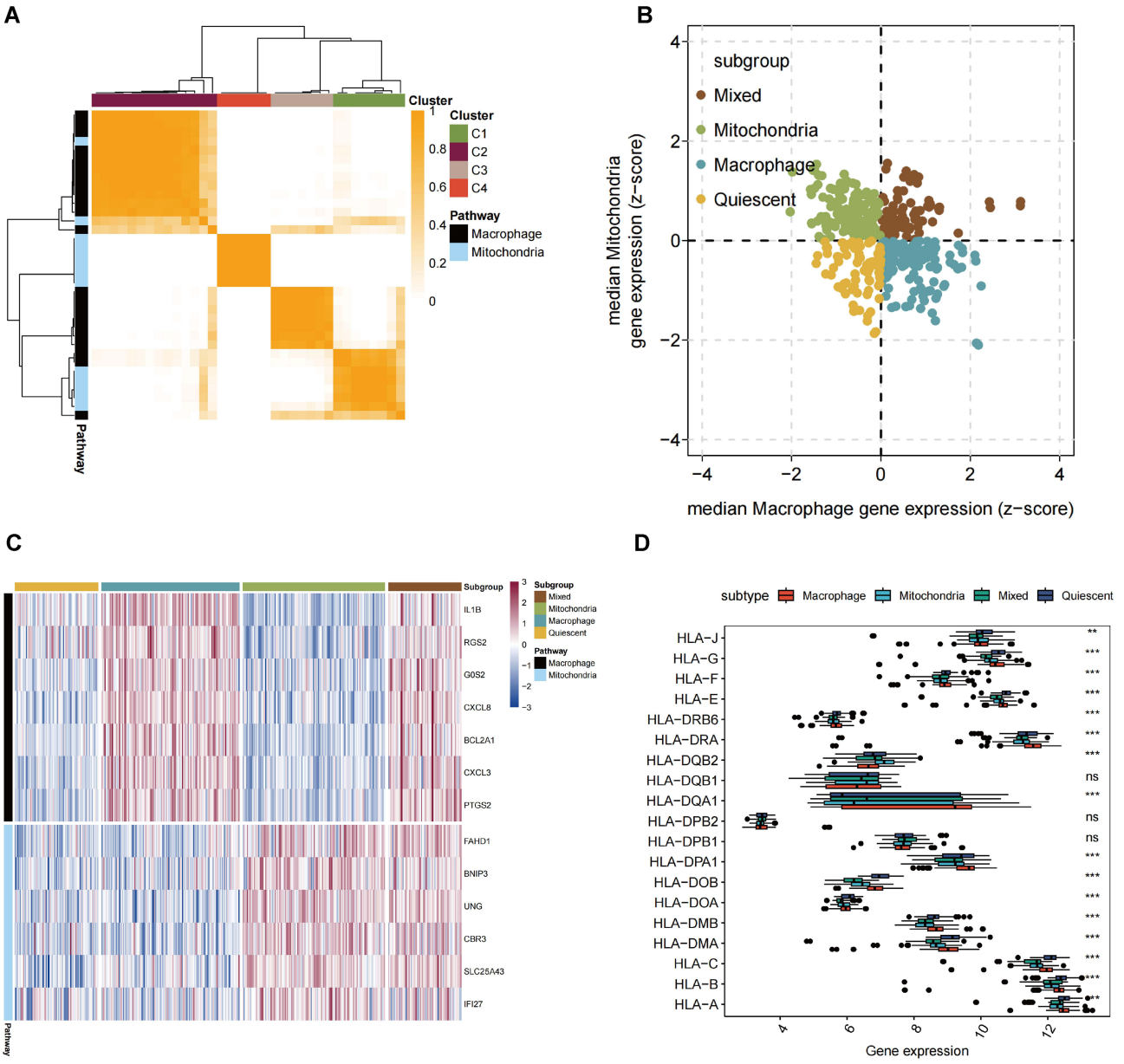

Figure 3 Molecular subtypes in periodontitis (**A**) Consensus clustering matrix identifying four molecular subtypes. (**B**) Scatter plot illustrating median expression of macrophage- and mitochondria-related genes across subtypes. (**C**) Heatmap showing expression patterns of key genes across subtypes. (**D**) Box plots depicting variations in HLA gene expression among subtypes.

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The biological significance of each subtype is as follows:

a. Quiescent subtype: Low expression of both macrophage and mitochondrial gene sets.
b. Macrophage-dominant subtype: High expression of macrophage-related genes, including LYZ, IL1B, and CXCL8, and low expression of mitochondrial-related genes, indicating strong immune activity.
c. Mitochondria-dominant subtype: High expression of mitochondrial-related genes, such as FAHD1 and BNIP3, and low expression of macrophage-related genes, reflecting significant mitochondrial dysfunction.
d. Mixed subtype: High expression of both macrophage and mitochondrial-related genes, suggesting concurrent immune activation and mitochondrial involvement.

This integrative analysis highlights the key interplay between immune responses and mitochondrial dysfunction, providing new insights into the molecular heterogeneity of periodontitis.

The heatmap in Figure 3C visualizes the expression levels of co-expressed macrophage and mitochondrial genes across these subtypes, providing a clear depiction of the gene expression differences between them. Furthermore, the immunological characteristics of these subtypes, including the differential expression of immune-related genes such as HLA-DQA1, HLA-DPB1, and HLA-DRA (Figure 3D), highlight the biological diversity among periodontitis patients. The Macrophage-dominant subtype exhibited elevated levels of these HLA genes, indicating enhanced immune activity in this group compared to other subtypes. Significant variations in other HLA genes, including HLA-B and HLA-C, were also observed, further underscoring the distinct immunological profiles of the identified subtypes.

Characterization of Immune Microenvironment in Periodontitis Subtypes

With the molecular subtypes identified, the immune microenvironment associated with each subtype was characterized to understand their functional implications. The expression of key immune-related genes and the composition of immune cell populations were analyzed for each subtype.

Figure 4A displays violin plots showing the expression distribution of various immune-related genes across the four subtypes. These genes include markers for T cells (CD3E, CD4, CD8A), macrophages (CD68, IL1B), and other immune cells, such as neutrophils and NK cells (NKG7). Distinct patterns of gene expression were observed among the subtypes, indicating differential immune activation states. For example, the Macrophage-dominant subtype exhibited elevated expression of CD68 and IL1B, markers associated with pro-inflammatory macrophages, while the Mitochondria-dominant subtype displayed higher expression of NKG7, indicative of NK cell activity (Figure 4B).

To provide a comprehensive view of the immune landscape, the relative proportions of key immune cell types for each subtype were calculated using established gene signatures, as depicted in Figure 4C. This analysis revealed that the Macrophage-dominant subtype exhibited a higher proportion of macrophages (both M1 and M2 subtypes) and T cells, whereas the Mitochondria-dominant subtype showed an elevated proportion of NK cells. The Mixed subtype displayed a more diverse immune cell composition, with no single cell type predominating, suggesting a more balanced immune response compared to the other subtypes. In contrast, the Quiescent subtype generally showed lower proportions of all immune cells examined, suggesting a suppressed or inactive immune state.

Machine Learning Approaches Identify Key Diagnostic Biomarkers

After identifying 13 potential marker genes through preliminary analysis, multiple machine learning algorithms were applied to refine the set of key diagnostic biomarkers. Specifically, LASSO regression, SVM-RFE, Random Forest, Boruta, and XGBoost were employed to evaluate the importance of each gene based on different selection criteria. These algorithms provide different perspectives on gene selection, such as feature reduction (SVM-RFE) and regularization (LASSO), ensuring a comprehensive evaluation of the diagnostic potential of the genes.

The multi-method approach ensured that the most robust markers were consistently selected across different algorithms. As shown in Figure 5B, the Venn diagram illustrates the overlap of genes identified by the five machine learning methods. Out of the initial 13 genes, 8 key genes were consistently selected by at least three different algorithms, underscoring their importance. These genes include IL1B, CXCL8, G0S2, FAHD1, BNIP3, UNG, CBR3, and

SLC25A43. Notably, IL1B and CXCL8 were identified across all five methods, reinforcing their significance as top diagnostic markers.

These 8 genes consistently demonstrated stable diagnostic potential across multiple algorithms, further validating their reliability as robust diagnostic biomarkers for periodontitis. The importance of these 8 genes was further validated through feature importance rankings provided by Random Forest and XGBoost (Figure 5A), which confirmed that IL1B and CXCL8 had the highest feature importance scores. Additionally, ROC curve analysis (Figure 5C) was used to quantify the diagnostic performance of these genes. IL1B exhibited the highest discriminative power with an AUC of 0.846, followed closely by CXCL8 with an AUC of 0.784. The remaining six genes, including BNIP3 and UNG, also demonstrated good diagnostic potential, with AUC values ranging from 0.769 to 0.899.

Gene Expression Profiles Across Cell Types and Molecular Subtypes

Violin plots demonstrate the differential expression of IL1B, G0S2, CXCL8, FAHD1, BNIP3, UNG, CBR3, and SLC25A43 between periodontitis cases and control samples. IL1B, G0S2, and CXCL8 show significantly higher expression levels in periodontitis samples, suggesting their involvement in pro-inflammatory processes. Conversely, FAHD1, BNIP3, and UNG are notably downregulated in periodontitis tissues, potentially indicating their roles in mitochondrial dysfunction and immune dysregulation. SLC25A43 and CBR3 also present elevated expression in periodontitis, though the magnitude of change is less pronounced compared to other genes (Figure 6A).

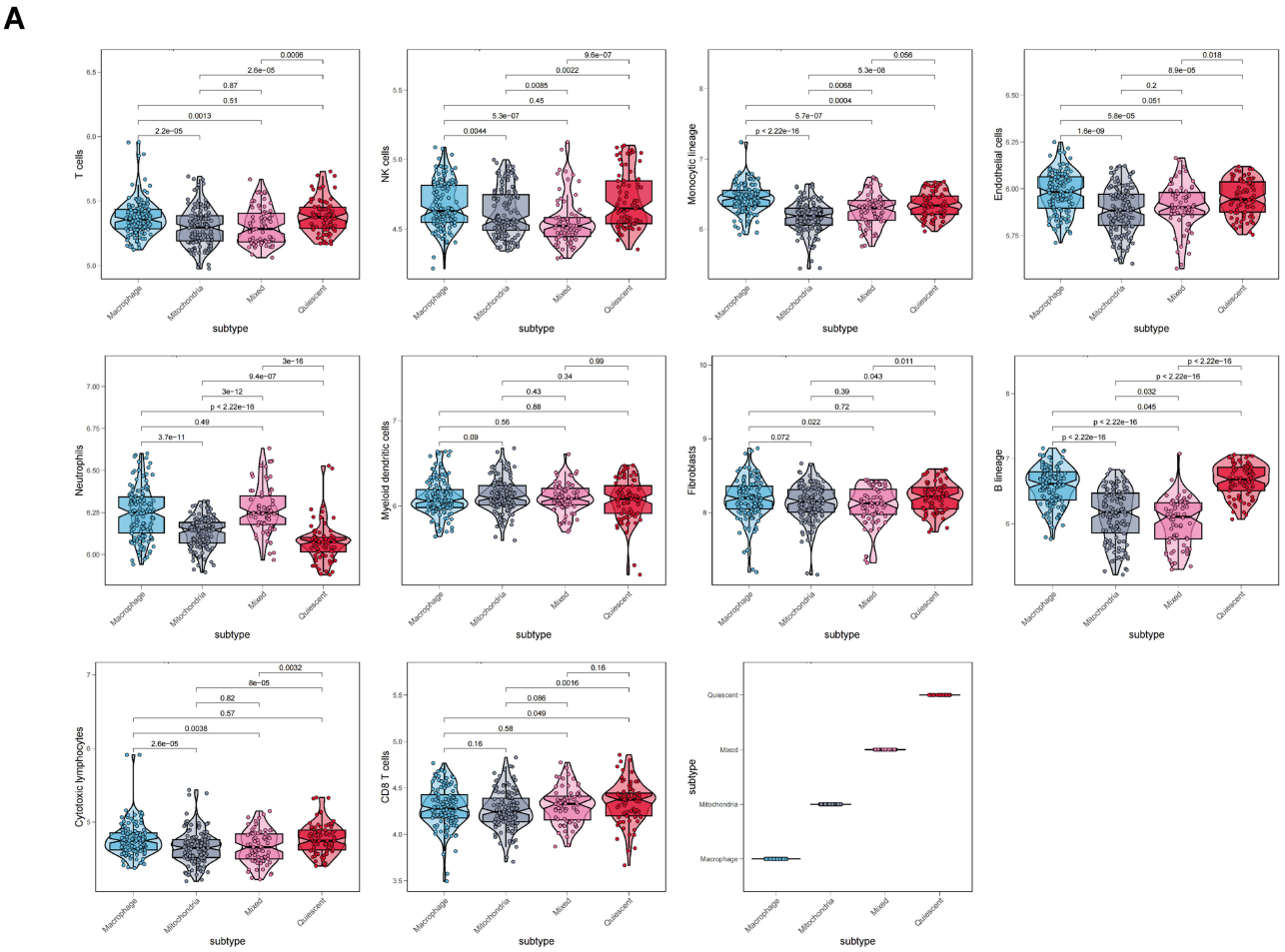

Figure 4 Continued.

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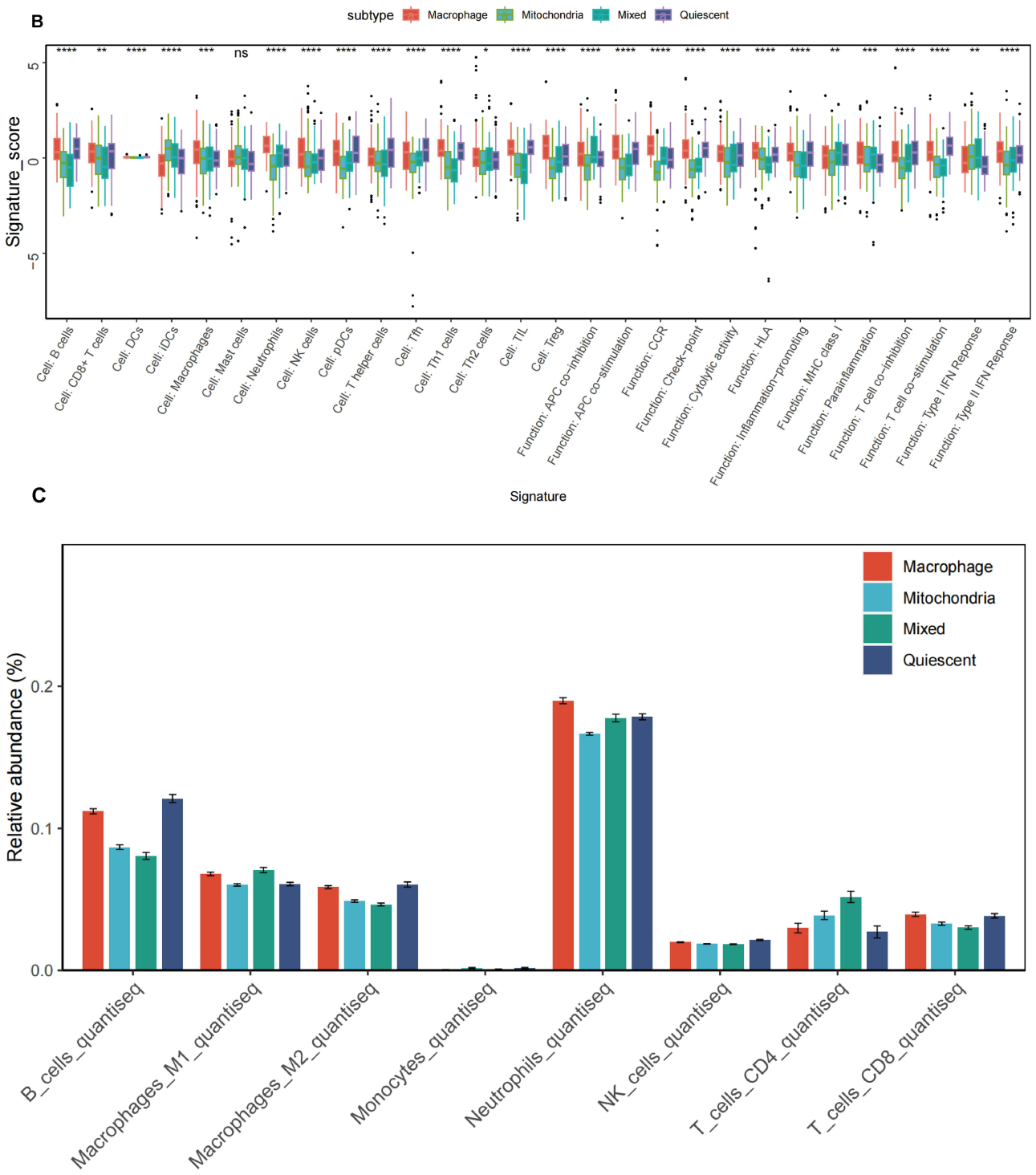

Figure 4 Immune microenvironment characteristics of periodontitis molecular subtypes. (**A**) Violin plots illustrating the expression of immune-related genes across four molecular subtypes. (**B**) Box plots showing differences in immune cell proportions between subtypes. Statistical significance is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001,****p < 0.0001. (**C**) Stacked bar plot presenting the relative abundances of key immune cell types in each subtype.

Figure 6B further explores gene expression across the four molecular subtypes: mitochondria-dominant, quiescent, macrophage-dominant, and mixed. Expression patterns vary distinctly among these subtypes. FAHD1 and BNIP3 are predominantly downregulated in the mitochondria-dominant subtype, reinforcing their association with mitochondrial dysfunction in periodontitis. IL1B and CXCL8 are highly expressed in the macrophage-dominant subtype, reflecting their

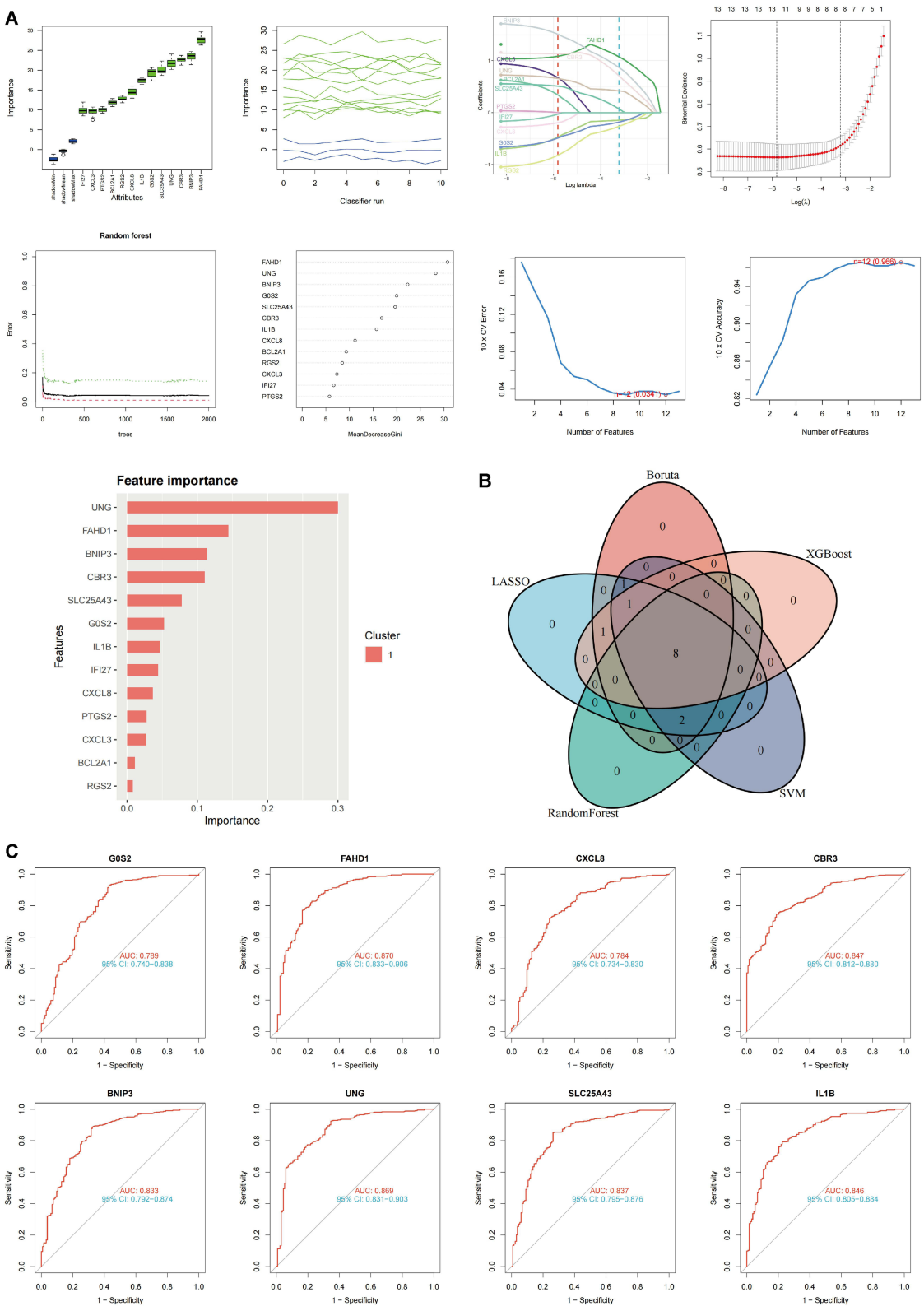

Figure 5 Key diagnostic biomarkers identified by machine learning approaches. (**A**) Outputs from various machine learning models: LASSO regression (top left), SVM-RFE (top middle), Random Forest and XGBoost (top right and bottom right), and Boruta (bottom left). (**B**) Venn diagram showing overlapping biomarkers identified across five machine learning models. (**C**) ROC curves demonstrating the diagnostic performance (AUC) of the selected biomarkers.

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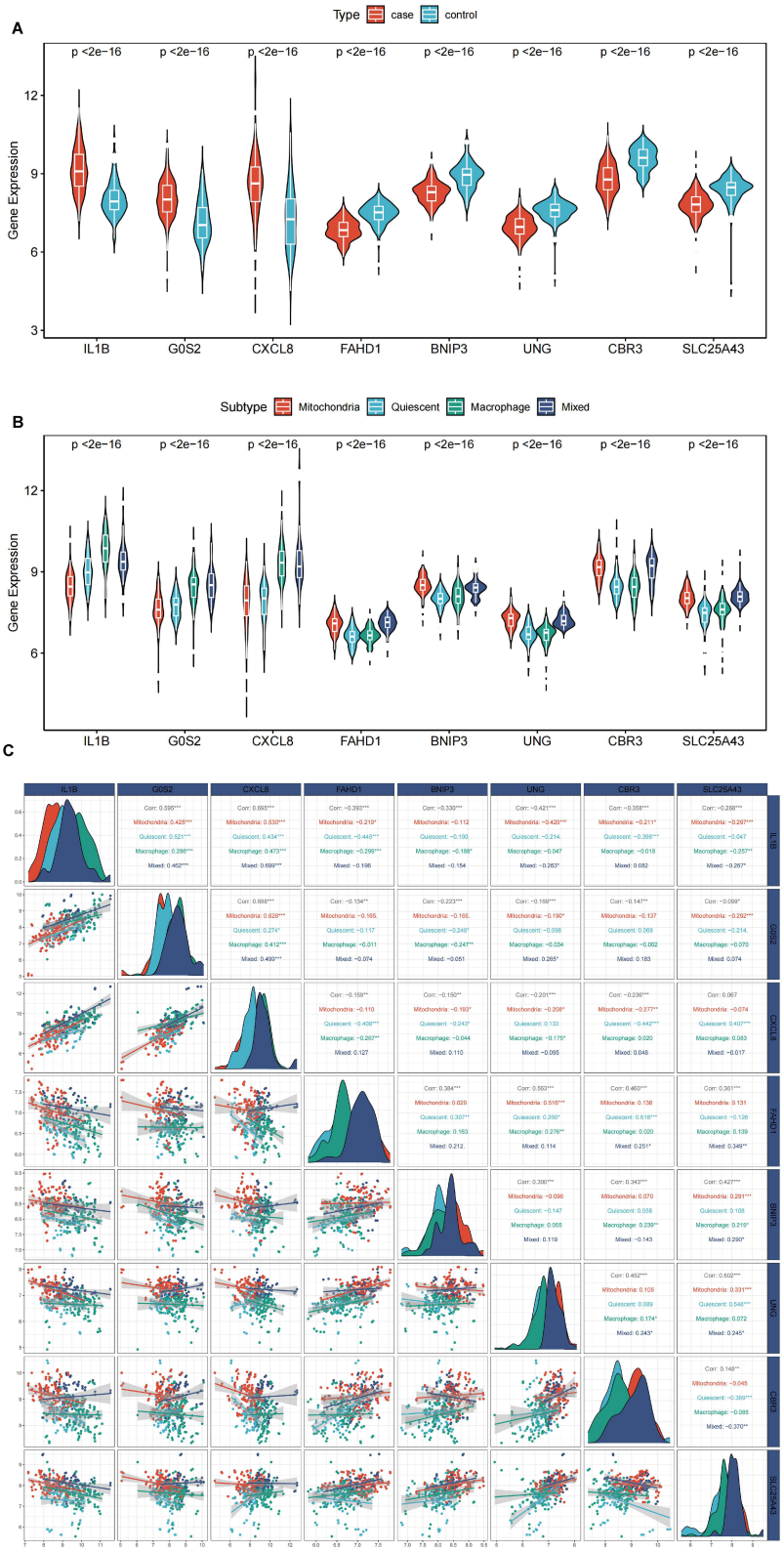

Figure 6 Expression profiles of key genes in periodontitis cases and molecular subtypes. (**A**) Violin plots illustrating the differential expression of key genes between periodontitis cases and controls. (**B**) Violin plots showing gene expression across four molecular subtypes. (**C**) Correlation matrix of key genes, with color intensity representing the strength and direction of correlations. Statistical significance is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

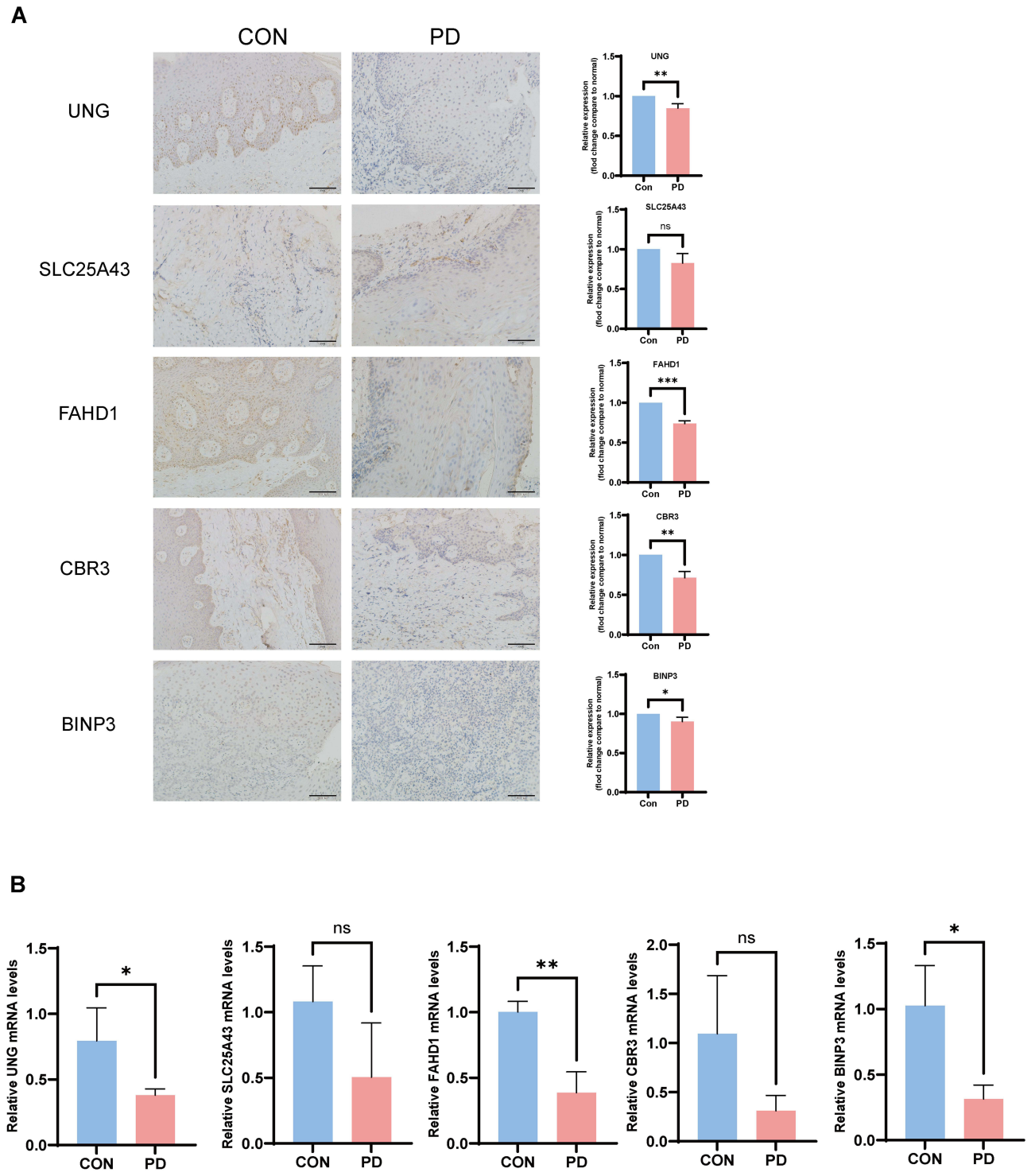

Figure 7 Validation of key genes in clinical samples. (**A**) Immunohistochemistry (IHC) staining of UNG, SLC25A43, FAHD1, CBR3, and BNIP3 in control (CON) and periodontitis (PD) tissues. Quantification of staining is shown on the right. Scale bars = 100 μm. (**B**) Quantitative PCR analysis of mRNA levels for the same genes in CON and PD tissues. Data are shown as mean ± SEM. ns: not significant; *p < 0.05, **p < 0.01, ***p < 0.001.

central role in the inflammatory response. The mixed subtype exhibits elevated expression of both macrophage and mitochondrial-related genes, indicating a more complex interaction between inflammation and mitochondrial function.

Correlation analyses highlight the relationships between these genes across different subtypes. Strong positive correlations between IL1B and CXCL8 are observed, particularly in the macrophage-dominant subtype, underscoring

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their co-regulation in driving inflammation. In contrast, FAHD1 and BNIP3 exhibit negative correlations with inflammatory markers, especially within the mitochondria-dominant subtype, suggesting their potential role in mitigating oxidative stress and mitochondrial damage. SLC25A43 and CBR3 display weaker correlations with other genes, which may indicate their less central roles in the molecular mechanisms of periodontitis (Figure 6C).

Validation of Key Genes in Clinical Samples

Immunohistochemistry (IHC) and quantitative real-time PCR (qRT-PCR) analyses were performed on periodontitis (PD) and healthy control (CON) samples to validate the expression patterns of identified key genes (Figure 7).

IHC staining revealed the expression patterns of UNG, SLC25A43, FAHD1, CBR3, and BNIP3 in periodontal tissues (Figure 7A). Quantitative analysis showed significantly decreased expression of UNG ($p < 0.01$), FAHD1 ($p < 0.001$), and CBR3 ($p < 0.01$) in PD samples compared to controls. No significant differences were observed in the expression levels of SLC25A43 and BNIP3 between PD and control samples based on IHC results.

qRT-PCR analysis (Figure 7B) confirmed the IHC results for UNG and FAHD1, showing significantly lower mRNA levels in PD samples ($p < 0.05$ and $p < 0.01$, respectively). However, BNIP3 mRNA levels were significantly higher in PD samples ($p < 0.05$), differing from the IHC results, suggesting possible post-transcriptional regulation or differences in protein stability. CBR3 showed a trend towards lower expression in PD samples, though this did not reach statistical significance, which may still indicate a potential role in periodontitis pathogenesis that requires further investigation. SLC25A43 mRNA levels showed no significant difference between groups, consistent with the IHC results.

Discussion

This study provides a systematic exploration of the molecular heterogeneity and key biomarkers in periodontitis by integrating single-cell RNA sequencing, whole transcriptome data, and machine learning. Our research is among the first to combine mitochondrial dysfunction with immune responses, revealing the complex pathogenesis of periodontitis and identifying distinct molecular subtypes,[31] which lay the groundwork for personalized diagnostic and therapeutic strategies. Specifically, our integrative analysis revealed distinct molecular subtypes, identified eight key diagnostic biomarkers (such as IL1B, CXCL8, FAHD1, and BNIP3), and demonstrated significant associations between mitochondrial dysfunction and immune dysregulation, providing novel insights into periodontitis pathogenesis.

These findings not only advance the understanding of periodontitis molecular pathogenesis but also suggest pathways for improving clinical treatment. The integration of molecular diagnostics with conventional therapies could enhance treatment precision, reduce disease recurrence, and improve patient outcomes. Such strategies hold potential for improving quality of life by minimizing functional impairments and fostering a more patient-centered approach in periodontal care. Future validation in longitudinal and multicenter trials is essential for broader clinical applicability.

To further understand the specific mechanisms underlying these findings, we analyzed the gene expression profiles and immune cell composition of the identified subtypes. Four primary molecular subtypes were identified: macrophage-dominant, mitochondria-dominant, quiescent, and mixed. These subtypes showed significant differences in gene expression profiles and immune cell composition. For example, the macrophage-dominant subtype displayed elevated expression of IL1B and CXCL8, indicating a heightened inflammatory response.[32,33] In contrast, the mitochondria-dominant subtype exhibited significant downregulation of FAHD1 and BNIP3, suggesting that mitochondrial dysfunction plays a crucial role in disease progression.[34,35]

FAHD1 is a key mitochondrial enzyme regulating cellular energy metabolism,[36] and its downregulation likely leads to metabolic disturbances and increased oxidative stress, exacerbating tissue damage.[37] BNIP3, a critical regulator of mitophagy, is also downregulated, which may impair the removal of damaged mitochondria, leading to their accumulation, further exacerbating inflammation and tissue destruction. The failure to clear damaged mitochondria could lead to excessive production of reactive oxygen species (ROS) within the mitochondria, intensifying inflammation and oxidative damage in periodontal tissues. Our machine learning analysis identified FAHD1, BNIP3, UNG, CBR3, and SLC25A43 as potential biomarkers.

These genes displayed distinct expression patterns across the different molecular subtypes of periodontitis. The ROC curve analysis indicated that FAHD1 had an AUC of 0.85, BNIP3 an AUC of 0.82, and UNG an AUC of 0.79,

highlighting their strong diagnostic potential. Although CBR3 and SLC25A43 did not show significant expression changes in our clinical samples, they may still play regulatory roles under specific pathological conditions, particularly in certain subtypes.[38]

BNIP3 showed inconsistent results between qPCR and IHC. While qPCR analysis revealed significantly higher mRNA levels of BNIP3 in periodontitis tissues ($p < 0.05$), IHC did not show a corresponding increase in protein expression. This discrepancy could be due to the different sensitivities of the two methods. IHC measures protein levels, which can be influenced by post-translational modifications, degradation, and protein stability, while qPCR captures transcriptional activity at the mRNA level.[39] BNIP3 mRNA may be upregulated in response to inflammatory signals, but its protein could undergo rapid degradation or post-translational regulation, limiting its accumulation in tissues. Such mRNA-protein level discrepancies are not uncommon in inflamed tissues. Future studies using more sensitive protein detection methods, such as Western blot or mass spectrometry, could provide deeper insights into the regulation of BNIP3 at different molecular levels.

As for CBR3, although both IHC and qPCR showed a trend toward lower expression in periodontitis samples, the differences did not reach statistical significance. This could be due to the small sample size or sample heterogeneity. CBR3 is known to play a role in oxidative stress responses, and its activity might be more pronounced under specific pathological conditions or at later stages of inflammation. The lack of significant findings suggests that CBR3 expression may vary depending on disease severity or progression, or its role in periodontitis may be context-dependent. Larger cohort studies or investigations into CBR3's role across different stages of periodontitis could clarify its biological relevance.[40]

Mitochondrial dysfunction is increasingly recognized as a key factor in periodontitis pathogenesis.[18] Mitochondria are not only essential for cellular energy production but also play pivotal roles in regulating autophagy, apoptosis, and immune signaling pathways during inflammation. When mitochondrial function is impaired, excessive ROS production can occur, triggering oxidative stress and activating inflammatory signaling pathways, such as the NF-κB pathway,[41] which further aggravates tissue inflammation. Moreover, mitochondrial dysfunction may influence macrophage polarization, favoring the accumulation of M1 pro-inflammatory macrophages, thereby intensifying the inflammatory response.[42]

This aligns with our findings in the mitochondria-dominant subtype, where mitochondrial gene dysregulation correlates with enhanced inflammation.[43] Compared to other recent molecular studies on periodontitis, our research offers new perspectives. Previous studies have mainly focused on immune responses and cytokine levels in periodontitis, with less attention given to the role of mitochondrial dysfunction. Our study, by integrating single-cell RNA sequencing and transcriptome data, highlights the regulatory mechanisms involving mitochondrial function in periodontitis. Additionally, while traditional studies often employ conventional gene expression analysis, our use of machine learning to systematically screen for key genes enhances the precision of biomarker identification. For instance, the identification of BNIP3 in our study not only associates it with autophagy but also points to its critical role in regulating inflammation via mitochondrial pathways.[44,45]

Our findings are consistent with recent studies that emphasize the role of mitochondrial damage and oxidative stress in periodontitis, reinforcing the importance of targeting mitochondria in disease management. Beyond periodontitis, these findings have broader implications for understanding other inflammatory and degenerative diseases. Mitochondrial dysfunction and dysregulated immune responses are hallmark features of various chronic inflammatory conditions, including rheumatoid arthritis, inflammatory bowel disease, and neurodegenerative disorders. The molecular mechanisms and biomarkers identified in this study, particularly FAHD1, BNIP3, and other mitochondrial-related genes, may provide valuable insights for developing diagnostic tools and targeted therapies applicable to these conditions. The broader applicability of these findings underscores the potential for translating periodontitis-specific discoveries into therapeutic strategies for other inflammatory diseases with similar pathogenic mechanisms. Moreover, the integrative analytical approach used in this study, combining single-cell sequencing and machine learning, could serve as a robust model for biomarker discovery in other complex diseases.

Although this study provides valuable insights into the molecular heterogeneity of periodontitis, further validation is necessary. The single-cell RNA sequencing data and transcriptome analysis revealed critical molecular features of the disease, but the precise functional roles of these biomarkers must be confirmed through additional experimental work.

Future studies should include larger, multi-center cohorts to validate the generalizability of these molecular subtypes and biomarkers. Longitudinal studies would also be beneficial for tracking the molecular changes over time, providing a clearer understanding of how these subtypes evolve during disease progression and their response to treatment.

From a clinical perspective, non-invasive testing methods based on these biomarkers, such as saliva or gingival crevicular fluid sampling, could be developed to provide an early diagnosis of periodontitis. Personalized treatment strategies can also be informed by these molecular subtypes. For instance, patients with the mitochondria-dominant subtype may benefit from antioxidant therapies or treatments aimed at improving mitochondrial function, whereas those with a macrophage-dominant subtype may respond better to immunomodulatory therapies.

The novelty of our study lies in the first-time integration of single-cell RNA sequencing with whole transcriptome data, revealing the complex interplay between mitochondrial dysfunction and macrophage immune responses in periodontitis. Through the application of machine learning algorithms, we systematically identified key genes and characterized molecular subtypes associated with periodontitis. This combination of multi-omics data and machine learning establishes a foundation for precision medicine in periodontitis and offers new perspectives for future diagnostic and personalized therapeutic strategies.

Limitations and Clinical Implications

This study provides significant insights into the molecular subtypes and biomarkers of periodontitis, yet challenges remain in translating these findings into clinical practice. A key limitation is the variability in immune responses among patients, influenced by factors such as genetics, environment, and comorbidities, which may affect the generalizability of biomarkers like IL1B and CXCL8. Future research should prioritize stratifying patients based on immune profiles and validating biomarkers across diverse populations to enhance their clinical relevance.

Moreover, biomarkers such as FAHD1, BNIP3, and IL1B hold great potential for non-invasive diagnostic methods, including saliva or gingival crevicular fluid sampling. These approaches can improve patient comfort, facilitate early detection, and support routine monitoring, reducing the burden of traditional diagnostic methods. Developing and validating these tools through further studies will be critical to ensuring their applicability in clinical practice.

However, while this study highlights the interplay between mitochondrial dysfunction and immune responses, it does not directly evaluate mitochondrial function within specific immune cell subtypes, particularly macrophages. This limitation underscores the need for future research focusing on mitochondrial dynamics, such as ROS production, mitophagy, and energy metabolism, within distinct immune cell populations. Addressing this gap through advanced techniques, such as live-cell imaging and mitochondrial function assays, will provide deeper mechanistic insights into periodontitis and support the development of more targeted therapeutic strategies.

Conclusion

This study systematically reveals the molecular heterogeneity in periodontitis, identifying distinct subtypes and key biomarkers related to mitochondrial dysfunction and inflammation. These findings provide a foundation for personalized diagnostics and therapeutic strategies. Importantly, integrating these biomarkers into non-invasive diagnostic tools could enhance patient comfort and compliance. Personalized treatments based on these molecular insights have the potential to improve clinical outcomes, reduce recurrence, and enhance the quality of life for periodontitis patients. Future validation studies are essential to translate these discoveries into clinical applications.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Kunming Medical University School and Hospital of Stomatology (Approval No. KYKQ2024MEC0065). All patients provided written informed consent before participation.

Consent for Publication

All authors agree to the publication of the article.

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Disclosure

All authors have no competing interest to declare for this research.

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