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Periodontitis biomarkers through thermal desorption-gas chromatography-mass spectrometry analysis

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Background: Periodontitis is a growing public health concern worldwide. Salivary volatile metabolites have emerged as promising biomarkers for the diagnosis of periodontal disease. However, research on the collection and identification of these metabolites in periodontitis patients remains limited.

Objectives: To explore methods for collecting and identifying salivary volatile metabolites in periodontitis patients and investigate their potential as biomarkers for diagnosing periodontal disease.

Method: Oral exhaled breath and saliva samples were collected from 115 periodontitis patients and 35 healthy individuals, divided into four cohorts. The discovery cohort (Periodontitis: $P = 55$, Healthy: $H = 23$) and the test cohort ($P = 48$, $H = 23$) were screened and validated for potential biomarkers in volatile metabolites from oral exhaled breath by thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS). The validation cohort 1 ($P = 12$, $H = 12$) was tested for volatile metabolites in saliva by solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS), while validation cohort 2 ($P = 55$, $H = 23$) was tested for metabolic pathways in saliva by liquid chromatography-mass spectrometry (LC-MS).

Result: A total of 78 Volatile organic compounds (VOCs) were detected by TD-GC-MS, with 14 differential VOCs identified. A diagnostic model was established using cyclohexanone, styrene, and ethanol, yielding a combined AUC of 0.8237. These metabolites were also detected in saliva by SPME-GC-MS, with cyclohexanone showing higher expression in the periodontitis group ($P < 0.05$). The caprolactam degradation pathway was a key source of volatile metabolites in the oral exhaled breath of periodontitis patients.

Conclusion: We developed a novel method for analyzing salivary volatile metabolites using TD-GC-MS, demonstrating potential for periodontitis diagnosis. Cyclohexanone is identified as a potential biomarker for periodontitis, and the caprolactam degradation pathway may play a significant role in future studies on oral microbiota dysbiosis in periodontitis patients.

KEYWORDS

salivary volatile metabolites, periodontitis, biomarker, TD-GC-MS, orally exhaled breath

1 Introduction

Periodontitis is a chronic infectious disease induced by dental plaque microorganisms, characterized by inflammation and destruction of periodontal supporting tissues, which may ultimately lead to tooth loss (1, 2). According to the Global Burden of Disease study, in 2017, severe periodontitis affected approximately 71.48 million people worldwide, with a disability-adjusted life year rate of 63.49 per 100,000 person-years. This represents a 6.01% increase since 1990, with a notably higher disease burden observed in Asia. Therefore, it is crucial to prioritize the prevention and management of periodontal disease (3).

In recent years, the utilization of biomarkers for early non-invasive diagnosis has become a prominent research focus (4–10). Volatile metabolites in saliva and oral exhaled breath, owing to their accessibility and completely non-invasive nature, demonstrate significant diagnostic potential (5, 11). Recent advances have demonstrated the significant potential of volatile metabolites as biomarkers for various systemic conditions, including respiratory diseases (such as lung cancer, asthma, and COPD) (12–14), digestive disorders (e.g., gastric diseases and Crohn's disease (15, 16), and endocrine disorders like obesity and diabetes (17), showing promise for improving disease diagnosis and developing novel biomarkers. The oral microbiota, particularly periodontopathogenic bacteria such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, produce characteristic volatile metabolites during their metabolic processes. *in vitro* studies have confirmed that these periodontal pathogens generate specific VOCs, including volatile sulfur compounds (e.g., methyl mercaptan and hydrogen sulfide), short-chain fatty acids, indole, and pyridine. These microbe-derived VOCs can diffuse from periodontal pockets into saliva and are subsequently released into oral exhaled breath, thereby providing a direct basis for non-invasive diagnostic approaches (18–20). One study also found elevated levels of volatile sulfur compounds (VSCs) and pyridine in the salivary volatile metabolites of periodontitis patients compared to healthy individuals (21). Saliva contains microorganisms and their metabolites, which interact with the body, and some of these metabolites are released as gases into the oral exhaled breath. A team of researchers has shown that Oral volatile metabolites involve a prominent oral source and that the potential impact of volatiles originating from the oral cavity should be considered in respiratory biomarker studies (22). However, there has been limited research on characterizing salivary volatile metabolites in oral exhaled breath of patients with periodontitis, highlighting the need for further investigation in this area.

The identification and analysis of volatile compounds primarily rely on gas chromatography (GC), mass spectrometry (MS), and their combined applications. Thermal Desorption (TD) is a widely used technique that utilizes a sorbent-containing device to capture and concentrate volatile metabolites before their introduction into the gas chromatograph. This method is particularly prevalent for collecting exhaled breath samples. During analysis, the sorbent is heated to enhance the volatility of the trapped compounds, facilitating their efficient separation and detection within the gas chromatograph (23). In this study, we analyzed oral volatile metabolites from periodontitis patients and healthy individuals using TD-GC-MS. Our objective was to explore methods for the collection and identification of salivary volatile metabolites in patients with periodontitis, and to investigate the potential of these metabolites as biomarkers for the diagnosis of periodontal diseases. Therefore, we modified the previous sampling method of exhaled breath, and further applied TD-GC-MS to the detection of salivary volatile metabolites, using the traditional solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) to validate the volatile metabolites of saliva, combined with the validation of relevant metabolic pathways by LC-MS.

2 Methods

2.1 Study population

The study was registered in Chinese Clinical Trial Registry (Registration Number: ChiCTR2300069047) on March 6, 2023 and was approved by the Local Ethics Committee of Peking University School of Stomatology and Stomatology Hospital (PKUSSIRB-202281149). Population inclusion criteria: a. Age 20–70 years old; b. Having at least 20 teeth in the mouth (excluding the third permanent molar) c. No systemic diseases; e. Signing informed consent; Population exclusion criteria: a. Presence of serious systemic diseases (kidney disease, rheumatoid arthritis, liver dysfunction, stroke or history of stroke) b. Being in the period of pregnancy or breastfeeding c. Presence of periapical abscesses, periodontal abscesses and other active oral infections; presence of oral mucosal disease; severe untreated caries in the mouth: wearing orthodontic appliances or removable dentures; d. History of antibiotic or immunization-related medication use in the past 3 months, e. History of periodontal therapy within 6 months.

2.2 Clinical examination and periodontal diagnosis

After obtaining a medical and dental history and a consent form, clinical examinations of all participants were performed by one specialized dentist using manual periodontal probes (PCPUNC 15; HuFriedy Mfg. Co., Inc., Chicago, IL, USA). The clinical periodontal indices, including probing depth (PD), and bleeding index (BI), were measured at six sites per tooth (mesio-

Abbreviations

VOCs, volatile organic compounds; VSCs, volatile sulfur compounds; GC, gas chromatography; MS, mass spectrometry; TD, Thermal Desorption; TD-GC-MS, Thermal Desorption-Gas Chromatography-Mass Spectrometry; SPME-GC-MS, Thermal desorption gas chromatography-mass spectrometry (); LC-MS, Liquid Chromatography-mass spectrometry.

buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual) (24). Subjects were included in different groups based on periodontal examination. Periodontitis patients was required to fulfill as diagnosis of stage II-III extensive periodontitis with reference to the 2018 International Consensus on New Classification of Periodontal and Periimplant Diseases. Healthy individuals was required to have all periodontal pockets probed at a depth of <4 mm and percentage of bleeding on probing $\leq 10\%$ (25).

2.3 Experimental method

We divided the included periodontitis patients and periodontally healthy individuals into four cohorts: discovery cohort, test cohort, validation cohort 1, and validation cohort 2. The discovery cohort and test cohort screened and validated the potential biomarkers in oral exhale breath by using TD-GC-MS. The validation cohort 1 further detected volatile metabolites in saliva by SPME-GC-MS. The validation cohort 2 tested for the metabolic pathway in saliva by LC-MS. Therefore, in our study, we developed a set of detection and validation methods that can be applied to the oral volatile metabolites of saliva (Figure 1).

2.4 Sample collection and preparation

Sampling was conducted between 9:00 and 11:00 a.m. Following an overnight fast, participants were required to abstain from all oral hygiene procedures, food and water intake, gum chewing, and smoking for at least 2 h prior to sample

collection. Immediately before sampling, subjects rinsed their mouths with water and subsequently rested for 10 min.

Participants were asked to collect 2–3 mL of non-irritating saliva. The whole saliva was stored without any processing for SPME-GC-MS analysis. The collected saliva was centrifuged (10,000 g, 4°C, 10 min), and the supernatant was retained for subsequent LC-MS analysis.

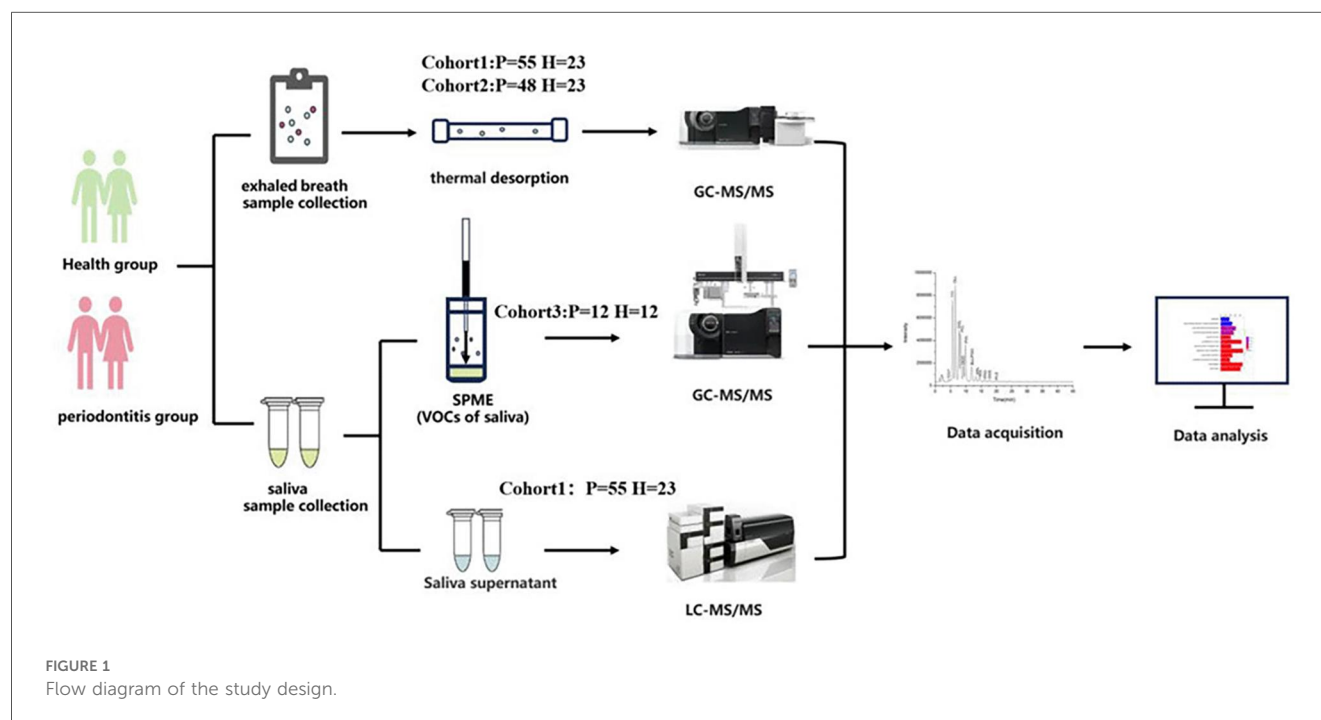
In addition, we have established a method for collecting the salivary volatile metabolites. Participants were instructed to swell cheeks to fill the mouth with gas as much as possible, wait for 10–15 s and then exhale all the gas into the Tedlar collection bag. The motion was repeated until the collection bag was full. Nasal inhalation was avoided prior to the exhaled gas collection.

Before collecting gas sample, the sampling bags were repeatedly cleaned by filling with nitrogen and heating to 60°C for 6 h to remove the residual contaminants. Each sample was transferred to the Tenax-TA trap tubes (Shimadzu, Japan) by pumping 800 mL from the sampling bag to the tube at 150 mL/min. The pumping process was completed by the mini-pump MP-W5P (Shimadzu, Japan). During this process, the VOCs in exhaled breath were trapped in the sorbent tubes. The tubes were sealed and stored at 4 °C until analyzed.

2.5 Laboratory analysis

2.5.1 Gas sample analysis by TD-GC-MS

To ensure data quality and control for analytical variability, all TD-GC-MS analyses incorporated Toluene-D8 as an internal standard, which was added to sample tubes prior to thermal



desorption for instrument response correction and data normalization, thus minimizing batch-to-batch variations.

The gas sample analysis was performed on GCMS-TQ8050 NX (Shimadzu, Japan) coupled with TD-30R thermal desorption (TD) instrument (Shimadzu, Japan). The trapped VOCs in the gas sample were desorbed at 260 °C under a flow of 60 mL/min for 5 min and adsorbed on a cold trap with a temperature of −20 °C. 2103;. Then the compounds were re-desorbed at 260 °C for 2 min and transfer to the GC inlet. The temperature of inlet and interface was 250 °C. 2103;. Chromatographic separation was performed on the SH-Rxi-624 fused silica capillary column (30 m × 0.25 mm × 1.4 μm, Shimadzu, Japan). Helium was used as the carrier gas with a constant linear velocity of 50 cm/s. The split ratio was 1:10. The GC temperature program was set as follows: the initial temperature was held at 35 °C for 5 min and ramped to 150 °C at a rate of 10 °C/min and to 260 °C at a rate of 30 °C/min with a hold time of 2 min. An electron ionization source was used with an ionization voltage of 70 eV and the ion source temperature was 250 °C. 2103;. The acquisition mode of data was Q3 scan mode with the m/z range 28–350. Chromatographic integration was performed by utilizing a single quantification ion extracted from the Extracted Ion Chromatogram (EIC). Raw GC-MS data were processed using GCMS solution software (Shimadzu, Japan) for peak integration and quantitative analysis for volatile metabolites in the sample. volatile metabolites were identified by spectral match according to the mass spectrometry library NIST 20-1, NIST 20-2 and NIST 20 s. The peak area was used to evaluate the relative concentrations of VOCs.

2.5.2 Saliva sample analysis by SPME-GC-MS and LC-MS

500 μL saliva sample was added to 20 mL headspace glass vial. The SPME-GC-MS analysis was performed on GCMS-TQ8050 equipped with AOC-6000Plus autosampler (Shimadzu, Japan). The SPME process were conducted by using 1.10 mm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME arrow. The saliva samples were incubated at 37 °C for 20 min and stirred continuously at 250 rpm. The SPME arrow was then exposed to the headspace for 45 min at 37 °C and desorbed at 250 °C in splitless mode for 5 min into the GC. The GC and MS conditions were set to the same as gas samples analysis.

100 μL salivary supernatant sample was added to a 1.5 mL centrifuge tube with 400 μL solution [acetonitrile:methanol=1:1(v:v)] containing 0.02 mg/mL internal standard (L-2-chlorophenylalanine) to extract metabolites. The samples were mixed by vortex for 30 s and low-temperature sonicated for 30 min (5 °C, 40 KHz). The samples were placed at −20 °C for 30 min to precipitate the proteins. Then the samples were centrifuged for 15 min (4 °C, 13,000 g). The supernatant was removed and blown dry under nitrogen. The sample was then re-solubilized with 100 μL solution (acetonitrile: water = 1:1) and extracted by low-temperature ultrasonication for 5 min (5 °C, 40 KHz), followed by centrifugation at 13,000 g and 4 °C for 10 min. The supernatant was transferred to sample vials for LC-MS/MS analysis. Details of the LC-MS laboratory tests for saliva samples are given in [Appendix 1](#).

2.6 Data analysis

Subject-related information as well as periodontal clinical parameters were analyzed by SPSS 24.0 software (SPSS; Chicago, IL, USA). Quantitative variables were described as mean ± standard deviation (SD), and frequencies or ratios were used for qualitative variables. The Student's t test was used to compare differences between groups, with a *p* value <0.05 defined as statistically significant. In addition, for gender results, the chi-square test was used to detect statistically significant differences. Differences between groups were analyzed by ANOVA, followed by Tukey's test. After performing a combined normalization of the discovery cohort and test cohort data, further data analysis was conducted. The SPME-GC-MS data were normalized using an internal standard method, followed by log10 transformation for analysis. The preprocessed matrix files were analyzed for differences. LC-MS and TD-GC-MS data results were analyzed by principal component analysis (PCA), ortho-least partial squares-discriminant analysis (OPLS-DA). The data matrix obtained by searching database was uploaded to the Majorbio cloud platform (<https://cloud.majorbio.com>) for data analysis. The performance of the model was screened and evaluated using Receiver Operating Characteristic curves (ROC). SPME-GC-MS results run on the MetaboAnalyst6.0 (<https://www.metaboanalyst.ca/>) platform. KEGG (https://www.kegg.jp/kegg/tool/map_pathway.html) pathway analysis was performed to identify the metabolic pathways associated with the differential and total metabolites.

3 Result

3.1 Demographic and clinical data

A total of 138 subjects were recruited from 2023 to 2024 at Peking University Stomatology Hospital, including a total of 115 periodontitis patients and 23 healthy individuals. They were divided into four cohorts as shown in [Table 1](#). There was no significant difference between the periodontitis group and healthy group in terms of the number of remaining teeth, gender (*p* = 0.191 and 0.853, respectively). It is statistically significant difference in age (*p* < 0.001). Regarding their periodontal clinical status, there was a statistically significant

TABLE 1 The characteristics of the study population.

Cohort	Periodontitis group		Healthy group		<i>p</i> -value
	PPD	<i>N</i>	PPD	<i>N</i>	
Discovery cohort	3.32 ± 0.12 ^a	<i>n</i> = 55	1.88 ± 0.40 ^b	<i>n</i> = 23	<0.001
Test cohort	3.39 ± 0.93 ^a	<i>n</i> = 48	1.88 ± 0.40 ^b	<i>n</i> = 23	<0.001
Validation cohort 1	3.33 ± 0.61 ^a	<i>n</i> = 12	1.84 ± 0.18 ^b	<i>n</i> = 12	<0.001
Validation cohort 2	3.32 ± 0.12 ^a	<i>n</i> = 55	1.88 ± 0.40 ^b	<i>n</i> = 23	<0.001

Data were described as mean ± SD. PPD, probing pocket depth; *N*: cohort number. Statistically significant difference (student's *t* test, *p*-value < 0.001); ^{a,b}Indicate statistically significant differences among groups (*p* < 0.05) as determined by one-way ANOVA followed by Tukey's HSD test.

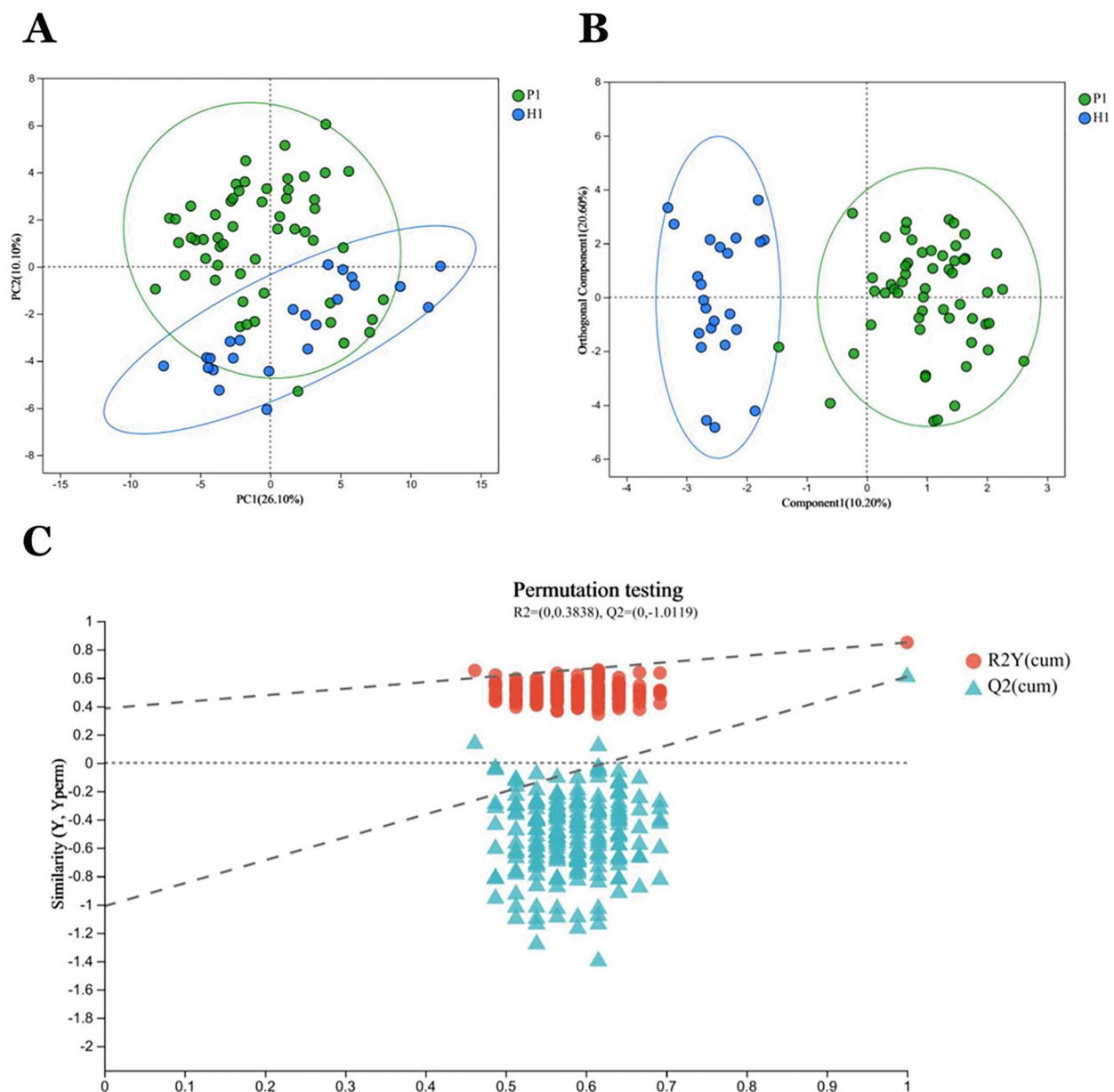


FIGURE 2

P1 and H1 group samples in general. (A) PCA analysis of sample relationships. Green dots indicate group P1 samples. Blue dots indicate group H1 samples. (B) OPLS-DA analysis of sample relationship. (C) Permutation testing of OPLS-DA: $R^2 = (0, 0.3838)$, $Q^2 = (0, -1.0119)$.

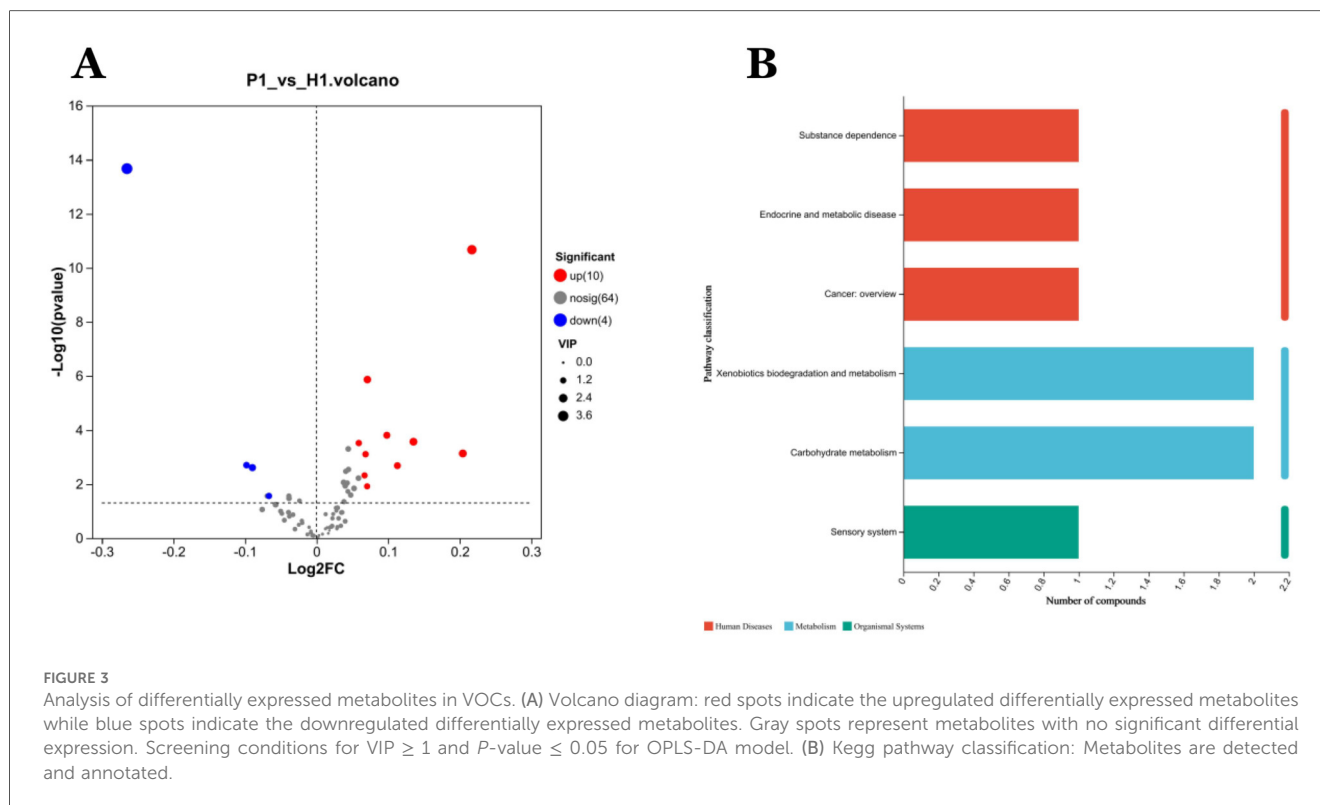
difference in periodontal probing depth (PPD), and gingival bleeding index (BI) (p value < 0.001).

3.2 Characterization of salivary volatile metabolites by TD-GC-MS

3.2.1 Analysis of gas samples

Principal Component Analysis (PCA) revealed distinct clustering patterns between sample groups P1 and H1 (Figure 2A). Intra-group samples demonstrated tight clustering,

indicating high experimental reproducibility within each group. Conversely, substantial intergroup separation suggested marked metabolic heterogeneity between the two groups. These metabolic differences were further confirmed by Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), which showed clear group discrimination (Figure 2B). The OPLS-DA model exhibited robust performance with high explanatory ($R^2Y = 0.85$) and predictive ($Q^2 = 0.608$) capabilities. To validate model integrity, a 200-iteration permutation test was performed, revealing a negative intercept (Q^2 regression line < 0) that effectively excluded overfitting concerns (Figure 2C).



3.2.2 Differential metabolite screening and pathway analysis

A total of 78 VOCs were identified in the discovery cohort. Differential VOCs were selected based on Variable Importance in Projection (VIP) scores from the OPLS-DA model and p -values from Student's t -test. VOCs with VIP > 1 and $P < 0.05$ were considered statistically significant (Figure 3A). A total of 14 differential VOCs (2-Ethyl-oxetane, Butanoic acid, methyl ester, 2-Pyrrolidinone, 1-methyl-, Ethanol, 2-butoxy-, Ethanol, Cyclopentane, methyl, Decane, 2,4-Dimethyl-1-heptene, Butylated Hydroxytoluene, Undecane, 3-methyl-, Cyclohexanone, 2,3-Butanedione, Styrene, Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester) were identified in our screening. To control for the potential confounding effect of age between the periodontitis group and the healthy control group, univariate linear regression analysis was conducted to assess the relationship between the 14 identified differential metabolites and age in both groups (detailed results are provided in Appendix 1). According to the pre-defined screening criteria ($R^2 > 0.5$ and $P < 0.05$), 2-Ethyl-oxetane ($R^2 = 0.537$, $P < 0.05$) and Cyclopentane, methyl ($R^2 = 0.512$, $P < 0.05$) in the healthy group were excluded due to significant age correlation. Ultimately, 12 metabolites showing no significant association with age were retained as candidate differential markers, including: Butanoic acid, methyl ester, 2-Pyrrolidinone, 1-methyl-, Ethanol, 2-butoxy-, Ethanol, Decane, 2,4-Dimethyl-1-heptene, Butylated Hydroxytoluene, Undecane, 3-methyl-, Cyclohexanone, 2,3-Butanedione, Styrene, Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester. As a result, 14 differential VOCs were identified and

subsequently mapped to KEGG pathways for further functional annotation. Among them, cyclohexanone, styrene, and ethanol were associated with the "Microbial metabolism in diverse environments" pathway (Figure 3B). Additionally, these three metabolites exhibited significantly higher expression levels in the periodontitis group compared to healthy controls (Figures 4C–E).

3.2.3 Diagnostic capability analysis of candidate differential metabolites

The three metabolites, cyclohexanone, styrene, and ethanol were evaluated by ROC (Figure 4A), as well as a joint ROC analysis was done to discriminate this group of metabolites (Figure 4B). The AUC of cyclohexanone was 0.7470 (95% CI: 0.6345–0.8596), the AUC of styrene was 0.6909 (95% CI: 0.5597, 0.8221), and the AUC of ethanol was 0.7478 (95% CI: 0.6069, 0.8887). The combined AUC of the three metabolites was 0.8237 (95% CI: 0.7975–0.85). The results indicated that the diagnostic model with the three metabolites together had well diagnostic effect.

3.2.4 Secondary validation of the diagnostic model

In the test cohort. The Random Forest (RF) model was used to identify the diagnostic ability of the diagnostic model composed of cyclohexanone, styrene, and ethanol. The results illustrated that when the RF model was constructed with the three metabolites, the model error rate was the lowest (Figure 5A), the AUC of the ROC curve was 0.9573 (Figure 5B), and the Random Forest model achieved a high level of accuracy, again demonstrating the superior diagnostic ability of the three metabolites.

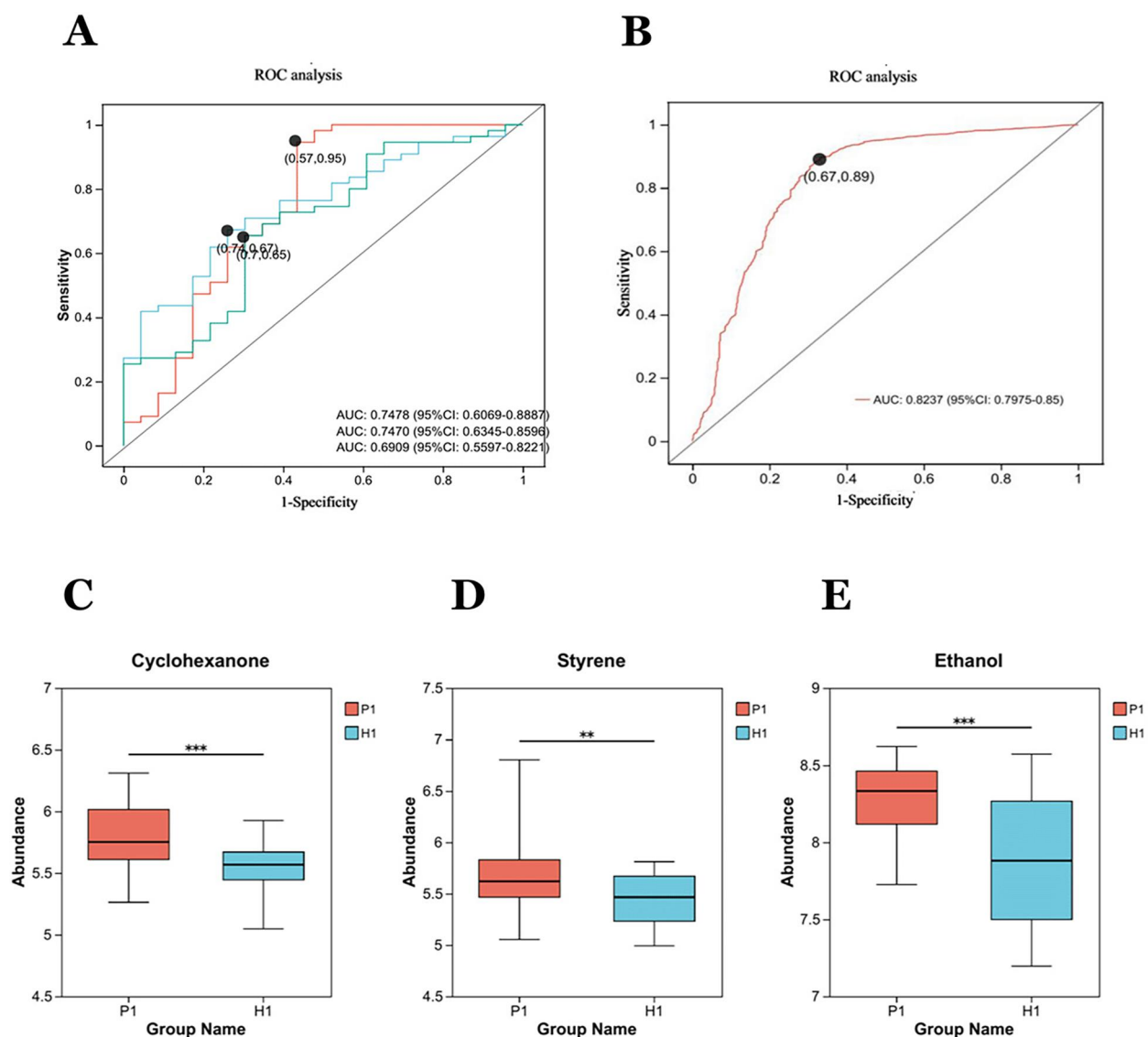


FIGURE 4

Diagnostic performance of exhaled gas metabolites cyclohexanone, styrene, ethanol, and combined combinations of in a discovery cohort: (A) independent receiver operating characteristic (ROC) analysis of cyclohexanone, styrene, ethanol. The areas under the curves (AUC) respectively are 0.7470, 0.6909, 0.7478. (B) The AUC for the combination of the three metabolites is 0.8237. (C–E) The box diagrams show the comparison of cyclohexanone, styrene, and ethanol the expression levels of between two groups. **Statistically significant difference (student's t test, p -value < 0.01). ***Statistically significant difference (student's t test, p -value < 0.0001).

3.3 Characterization of salivary volatile metabolites by SPME-GC-MS

A total of 39 salivary volatile metabolites were identified in Validation Cohort 1 by SPME-GC-MS. Principal Component Analysis (PCA) of the periodontitis group ($n = 12$) and healthy group ($n = 12$) revealed high within-group similarity and clear separation between groups, indicating distinct metabolic differences (Figure 6A). Based on the criteria of fold change (FC) ≥ 1.5 and $p < 0.05$, seven differential metabolites were identified: Sulfide, allyl methyl; 4-Heptanone; Disulfide, dimethyl; Decane, 2,2-dimethyl; Nonane; Cyclohexanone; and Hexanal (Figure 6B). Among these,

cyclohexanone was again identified as a differential metabolite, with its relative abundance significantly higher in the periodontitis group (Figure 6C).

3.4 Detection of metabolites in saliva by LC-MS

3.4.1 Analysis of saliva samples

PCA analysis revealed that the two groups of samples, S_P and S_H, exhibited relatively small distances within each group, indicating good reproducibility of the samples (Figure 7A). Significant differences between the groups

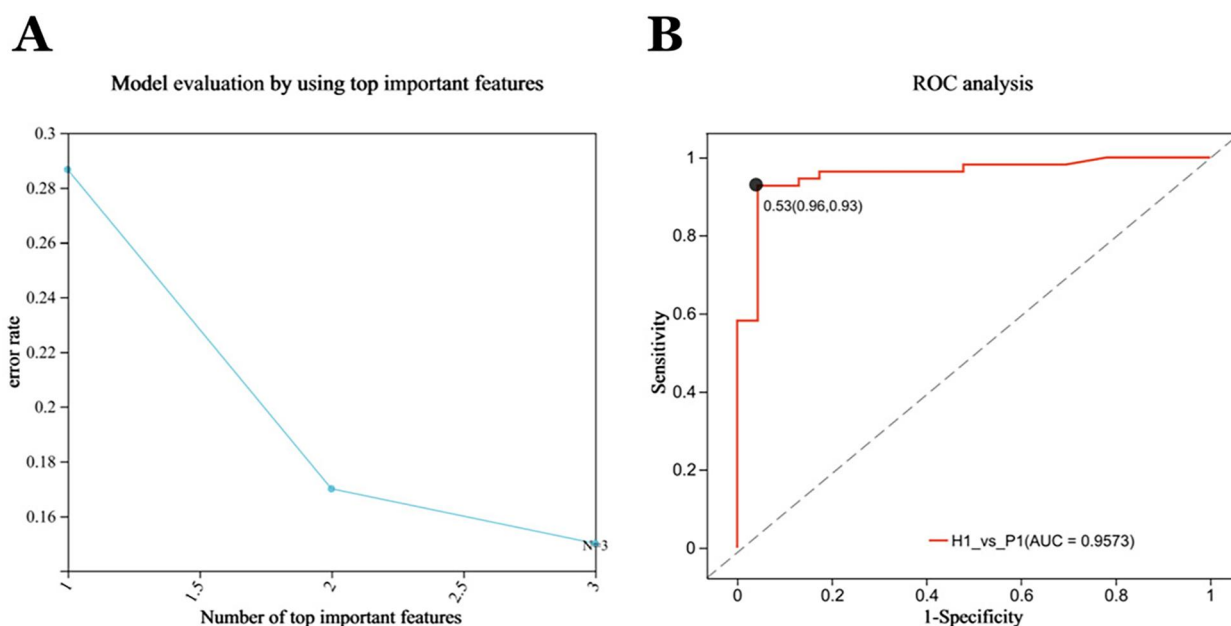


FIGURE 5

Forty-eight subjects were included in the test cohort for optimization, forming the validation cohort P2 ($n = 48$), H2 ($n = 23$). (A) Evaluation of Random Forest Model: The RFECV (Recursive Feature Elimination with Cross-Validation) algorithm was used to compute the features, with the horizontal coordinates representing the number of metabolites (variables) ranked TOP in terms of importance, and the vertical coordinates representing the average prediction error rate when the corresponding number of metabolites (variables) was used; solid points with markers in the plot indicate the point with the lowest error rate selected. The lowest model error rate was found when all three metabolites were modeled together. (B) The Independent Receiver operating characteristic (ROC) analysis of the RF models. The random forest models all achieved high accuracy with an AUC of 0.9573.

were observed in OPLS-DA analysis (Figure 7B). The degree of model explanation (R^2Y) and prediction (Q^2) were 0.922 and 0.552, respectively, indicating strong model performance. To ensure the reliability of the results, the OPLS-DA model was validated by a 200-permutation test. The intercept of the Q^2 regression line was -0.4492 , which is less than 0, confirming that the model was not overfitted (Figure 7C).

3.4.2 Differential metabolite screening and pathway analysis

A total of 1,844 metabolites were identified in saliva validation cohort 2. Differential metabolites were selected based on VIP scores from the OPLS-DA model and p -values from Student's t -test, with metabolites meeting the criteria of $VIP > 1$ and $p < 0.05$ considered statistically significant. This resulted in the identification of 480 differential metabolites (Figure 8A). Totally, 262 metabolites were mapped to various KEGG pathways, primarily related to lipid metabolism, amino acid metabolism, and microbial metabolism in different environments (Figure 8D). While styrene and cyclohexanone were detected in saliva, their differences between the periodontitis and healthy groups were not statistically significant ($p > 0.05$), and ethanol was not detected in saliva. Notably, cyclohexanone was mapped to the “map0930: caprolactam degradation pathway,” where six metabolites were detected: cyclohexane, cyclohexanone, 6-hydroxyhexanoic acid, N-cyclohexylformamide, adipic acid, and adipate semialdehyde.

Among these, N-cyclohexylformamide (Figure 8B) and adipic acid (Figure 8C) exhibited statistically significant differences between groups, with higher relative abundance in the saliva of periodontitis patients. As upstream and downstream metabolites of cyclohexanone, their elevated levels suggest that the caprolactam degradation pathway might play a role in the metabolic alterations observed in periodontitis patients (Figure 9).

4 Discussion

In this study, we analyzed the exhalation profiles of individuals with periodontitis and healthy controls from a discovery cohort by TD-GC-MS. After screening and validating a new cohort through machine learning with a “randomized forests” approach, we developed a diagnostic model consisting of cyclohexanone, styrene, and ethanol. These three metabolites were found to be present in the volatile metabolites of saliva, confirmed by SPME-GC-MS. Cyclohexanone exhibited a consistent trend in both saliva and exhaled breath, with statistically significant differences between the groups. Additionally, the analysis of salivary metabolites revealed a link between cyclohexanone and the caprolactam degradation pathway.

Previous studies have established volatile sulfur compounds (VSCs) as key contributors to oral malodor, often linked to Gram-negative anaerobic bacteria associated with periodontitis.

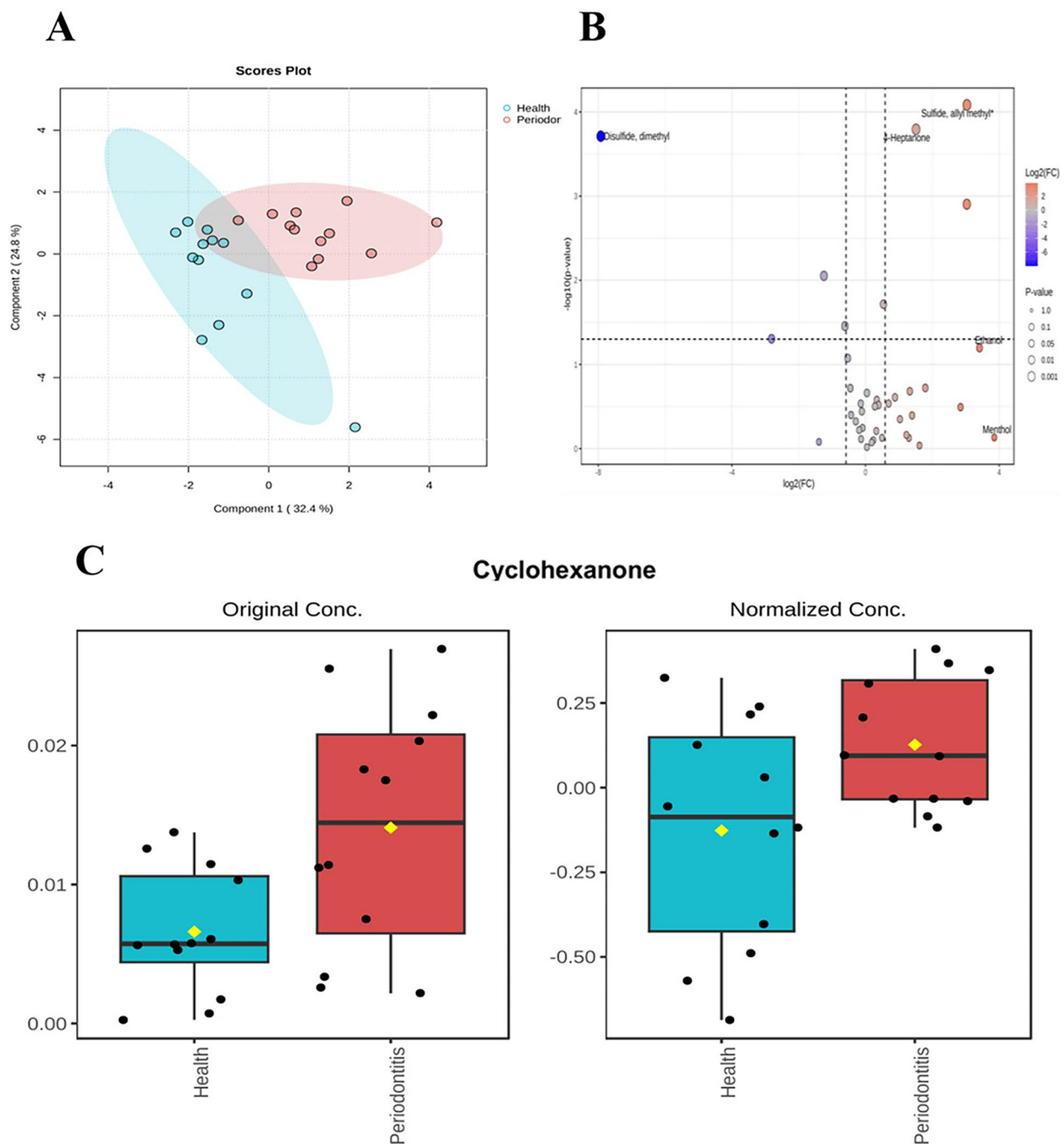


FIGURE 6

Differences in salivary volatile metabolites. saliva validation cohort 2: The sample consisted of a periodontitis group ($n = 12$) and a healthy group ($n = 12$). (A) Unsupervised principal component analysis (PCA) showed that the samples were reproducible within groups, but there were some differences between groups. (B) Volcano plot combines results from Fold Change (FC) Analysis and T-tests into one single graph, Mean quantitative differential fold change was limited to ± 1.5 , P value less than 0.05. (C) Histogram of cyclohexanone before and after standardized treatment.

VSCs have been proposed as potential biomarkers for periodontitis-related oral malodor in adults (26). In our study, we also observed a statistically significant increase in dimethyl sulfide concentration within the periodontitis group through SPME analysis volatile metabolites in saliva. However, no significant differences in sulfides were detected in the gas samples, possibly due to the concentration of sulfides being too

low in the gas samples or the presence of oxidation prior to sample processing. This finding is consistent with that of another research group, which observed a lower detection rate of volatile metabolites in exhaled breath compared to urine and other liquid samples (27). Previous research has suggested that periodontal disease is associated with an increased risk of halitosis. However, not all periodontitis patients exhibit oral

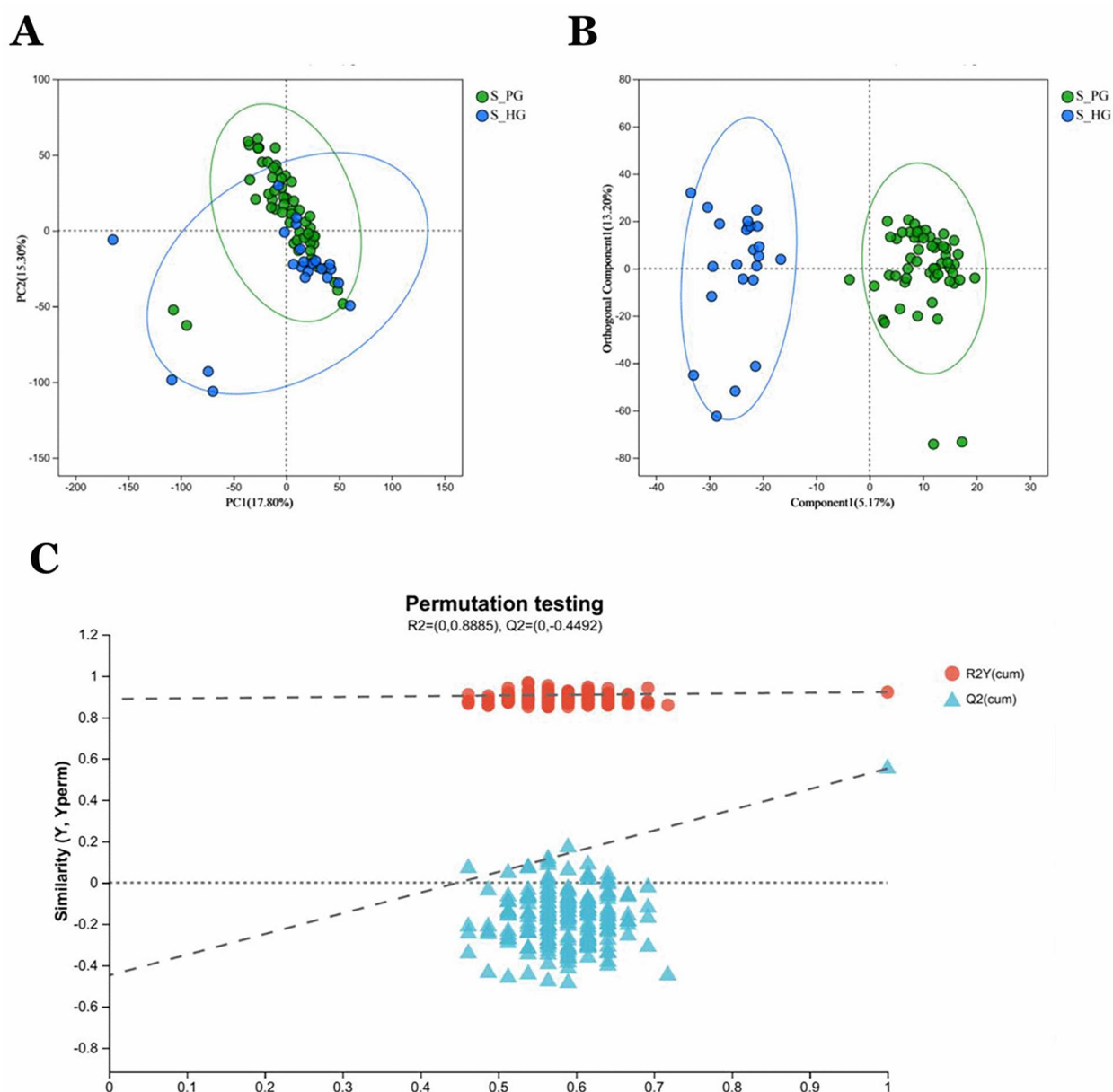


FIGURE 7

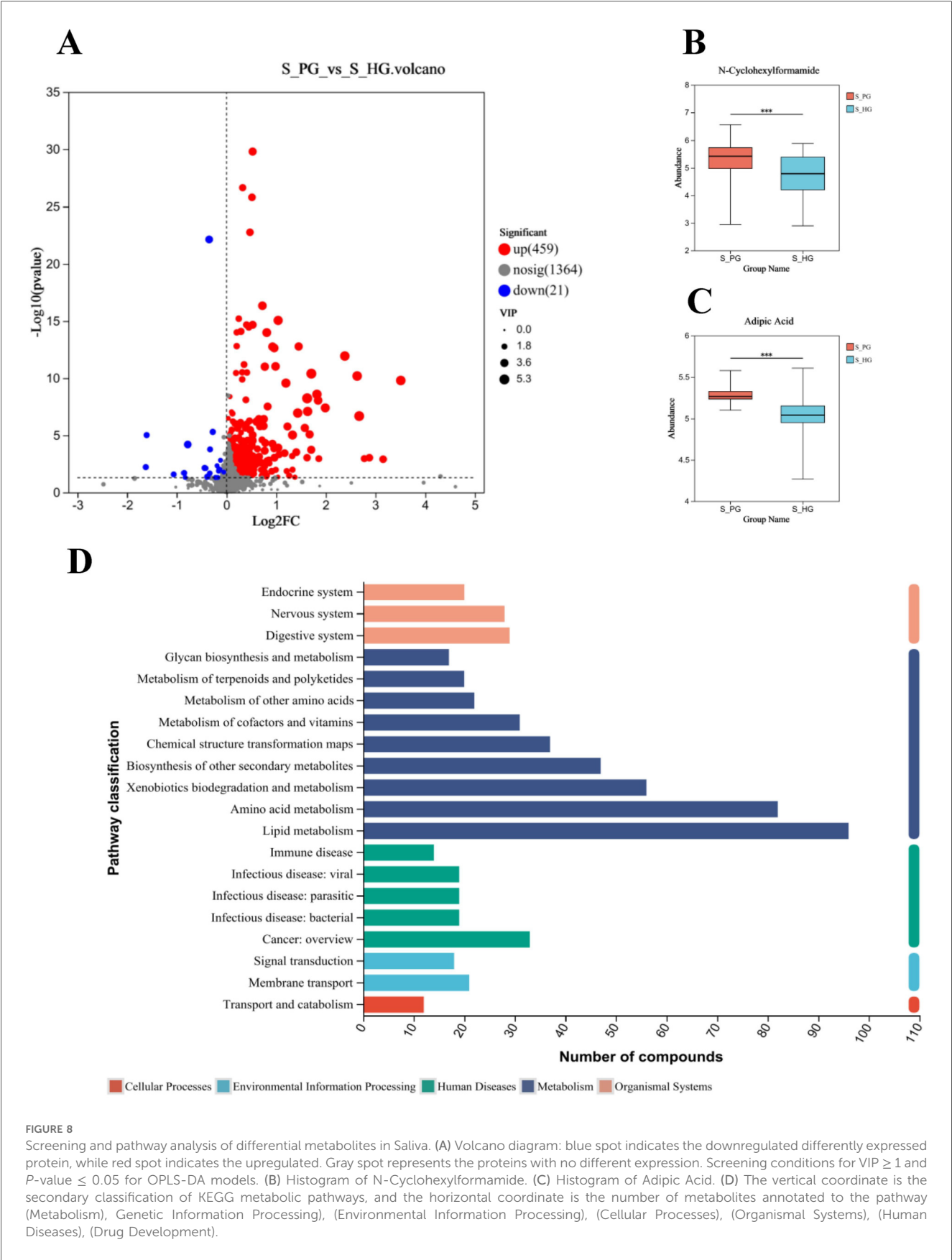
Saliva validation cohort 2: Saliva samples from periodontitis group ($n = 55$) and healthy group ($n = 23$) (A) PCA analysis of sample relationships. Green dots indicate S_P group, blue dots indicate S_H group. (B) OPLS-DA analysis of sample relationship. (C) Permutation testing of OPLS-DA: $R^2 = (0, 0.8885)$, $Q^2 = (0, -0.4492)$.

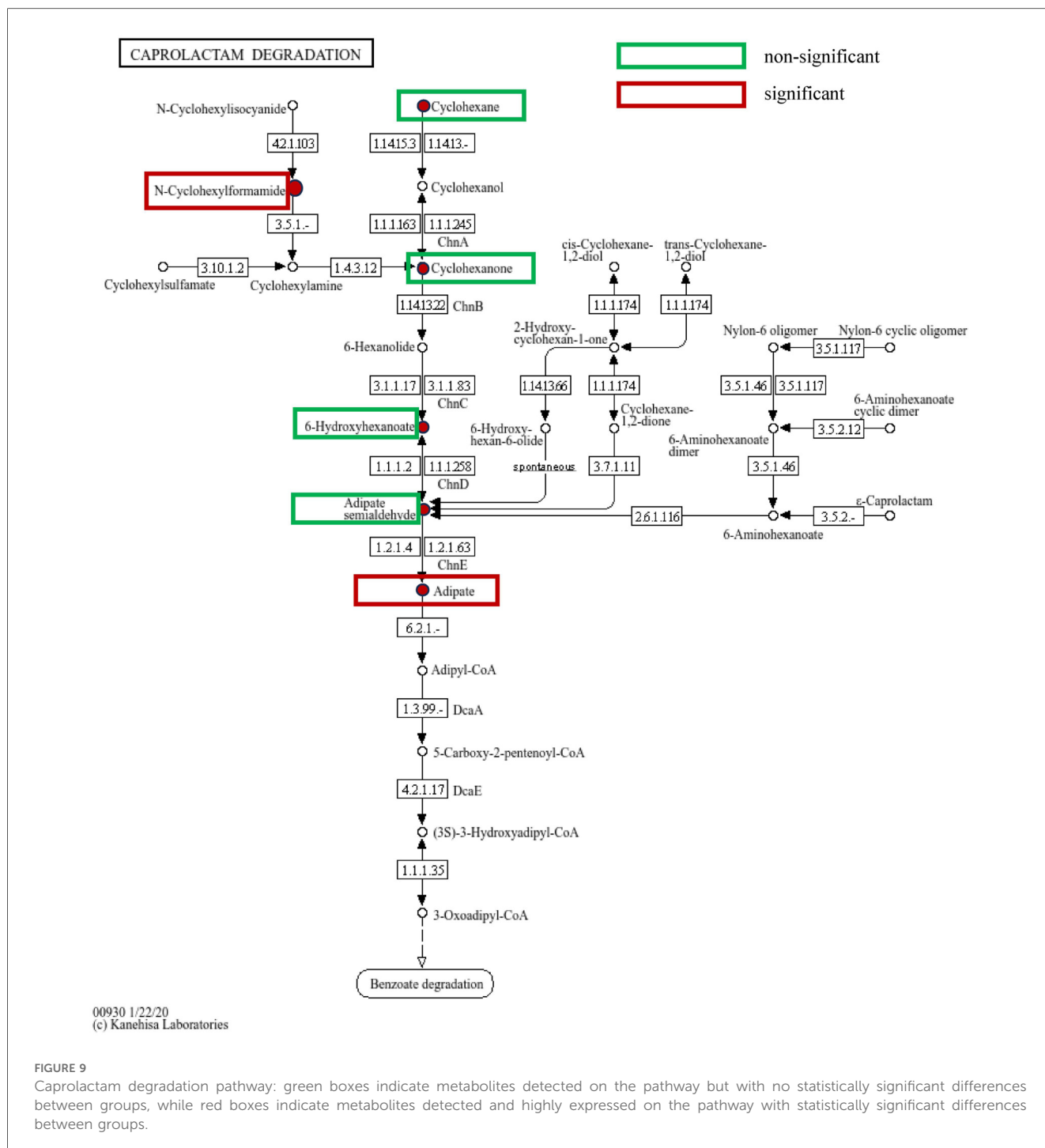
malodor, and VSCs are not directly applicable as biomarkers for periodontitis itself (28). Therefore, further investigation into the relationship between periodontitis and sulfides is warranted by improving the sampling methods and expanding the sample size.

In this study, ethanol is one of the volatile metabolites in the diagnostic model. Ethanol is a volatile organic compound in saliva, produced by bacterial alcohol dehydrogenase or alcohol consumption, and can be converted to acetaldehyde (29). In a previous study using nuclear magnetic resonance spectroscopy, ethanol was identified as a biomarker for periodontitis. Interestingly, the ethanol concentration was lower in periodontitis patients compared to healthy controls, suggesting a

link to microbial oxidative activity in periodontitis (5). This discrepancy may be attributed to differences in detection methods and the relatively low ethanol concentration in saliva. It is possible that ethanol is more likely to exist in the form of volatile metabolites in exhaled breath rather than in saliva.

Styrene, a volatile organic compound (VOC) that has been linked to increased lung cancer risk, has been extensively studied and identified as a promising biomarker for various health conditions (30). Recently, a research team employed needle trap extraction to analyze volatile compounds present above *Helicobacter pylori* cultures. They revealed that styrene was markedly elevated in the culture medium of *Helicobacter*





pylori. This phenomenon, along with other notably distinct metabolites, contributed to a unique volatile profile that could as a potential tool for monitoring infections caused by this pathogen (31). In our study, styrene levels were significantly elevated in the periodontitis group, suggesting a potential link to oral microbiome dysbiosis.

Cyclohexanone (CAS number: 108-94-1), a six-carbon cyclic compound with a ketone group, was identified as a potential volatile biomarker for periodontitis in this study. Cyclohexanone has been detected in previous research as a VOC associated with

various diseases. It has been found in the breath of patients with colorectal cancer (32), and elevated levels have been observed in patients with chronic kidney disease following hemodialysis (33). A research team had identified cyclohexanone as a promising biomarker in the breath of patients with biliary diseases, as well as in the volatile gases present in bile (34, 35). Our study excluded systemic diseases using strict inclusion criteria, suggesting that the elevated levels of cyclohexanone in periodontitis patients are primarily due to an imbalance in the oral microbiome. Another research team measured the components of VOCs by using

Proton Transfer Reaction Time-of-Flight Mass Spectrometry (MS). They found the cyclohexanone in oral volatile organic compounds will decrease by an average of 4.3% among healthy individuals after brushing their teeth every morning (36). It indicates that the detection rate of cyclohexanone will increase in the more complex oral microbiota environment, further demonstrating that patients with periodontitis have a dysbiosis of the oral microbiota, metabolic disorders, leading to an increase in cyclohexanone.

Our study revealed a significant increase in cyclohexanone concentration among salivary volatile metabolites in patients with periodontitis, with consistent detection through both TD-GC-MS and SPME-GC-MS analyses. Furthermore, the cyclohexanone was annotated to the caprolactam metabolic pathway along with the other five differential metabolite analysis of saliva. Therefore, we hypothesized that cyclohexanone in oral exhaled breath mainly originated from the caprolactam degradation pathway in saliva of periodontitis patients. The caprolactam degradation pathway has been regarded as an *ex vivo* metabolic pathway for different microbial metabolisms. However, in recent years, it has been found to play an important role in systemic diseases. We speculate that this metabolic pathway may also be an endogenous metabolic pathway. A research team analyzed the progression of nasal microbiota detection in young adults and elderly asthmatics using high-throughput sequencing, and found that the relative abundance of lysine degradation, N-glycan biosynthesis, caprolactam degradation, and PPAR signaling pathways was significantly lower in asthmatics than in non-asthmatics, which may be associated with the reduction of inflammation and degradation of air pollutants (37). In our research, N-Cyclohexylformamide had a rise in expression in the periodontitis group. It is at the upstream of the caprolactam degradation pathway. With the increase of metabolites at the upstream, the cyclohexanone in the downstream will also increase. Due to cyclohexanone slightly water-soluble and easily volatilizable properties, it is more often detected in the gaseous form.

The team of Kajsa Roslund employed a combination of solid-phase microextraction (SPME) and offline gas chromatography-mass spectrometry (GC-MS) to measure volatile compounds produced by bacteria. They analyzed the *in vitro* volatile fingerprints of several major pathogens associated with periodontitis, including *Porphyromonas gingivalis* ATCC 33277, *P. gingivalis* ATCC 53978 (W50), *P. gingivalis* OMG434, *Prevotella intermedia* ATCC 25611, *Prevotella nigrescens* ATCC 35563, and *Tannerella forsythia* ATCC 43037. Signals for ethanol and styrene were detected above cultures of these periodontitis-related microbes grown on agar medium. Specifically, the ethanol signal intensified at 90 h compared to 34 h for *P. gingivalis* W50 and *P. gingivalis* OMG434. While the styrene signal showed no significant variation above the three serotypes of *P. gingivalis*, it increased at 90 h for *P. nigrescens* ATCC 35563 and *T. forsythia* ATCC 43037. However, the study noted that these two volatile organic compounds (VOCs) are not necessarily produced directly by the bacteria, and the precise mechanisms were not investigated in detail. Although cyclohexanone itself was not directly detected in this study, propylcyclohexane was found to be produced by *P. gingivalis*

OMG434 (38). In a prior study by the same team utilizing Proton Transfer Reaction Time-of-Flight Mass Spectrometry (PTR-TOF-MS) to analyze these periodontitis-associated microorganisms, all three *P. gingivalis* strains appeared to produce high levels of acetone, suggesting its potential as a major marker for this bacterium (39). Ketone formation can be attributed to two possible pathways: the oxidation of secondary alcohols catalyzed either by alcohol dehydrogenase (ADH) or by cytochrome P450 CYP2E1 (40). Acetone can be generated via the ADH-catalyzed oxidation of 2-propanol, whereas the biosynthetic pathway for cyclohexanone involves a two-stage enzymatic reaction: initial hydroxylation of cyclohexane to cyclohexanol by bacterial cyclohexane monooxygenase, followed by further oxidation to cyclohexanone by cyclohexanol dehydrogenase. The present study identified the presence of cyclohexane in the saliva of periodontitis patients, along with significantly elevated concentrations of cyclohexanone in both exhaled breath and salivary volatile metabolites. We hypothesize that the dysbiosis induced by periodontitis may activate alkane metabolic pathways at the mechanistic level, thereby promoting the cyclohexane-cyclohexanol-cyclohexanone cascade. Notably, this metabolic axis coincides with key nodes of the caprolactam degradation pathway annotated in the KEGG database, suggesting that oral microbes might influence the host-microbe metabolic interaction network by modulating this pathway. These observations collectively provide a novel metabolic perspective for elucidating the pathological mechanisms of periodontitis and indicate that targeted intervention in the caprolactam degradation pathway could emerge as a crucial regulatory node for novel periodontitis treatment strategies.

This study has several limitations that warrant consideration. Firstly, strict screening conditions increased the difficulty of collecting healthy samples. The prevalence of subjects with periodontal health was 5% based on data from the Fourth National Oral Health Survey of China (41). It was the main reason for failing to match a sufficient number of periodontal health samples of older adults in this study. Secondly, while our metabolomic findings implicate the caprolactam degradation pathway in periodontitis, a key mechanistic limitation of our study is the inability to pinpoint the precise microbial sources of critical metabolites, such as N-cyclohexylformamide. The absence of supplementary microbial culture or metagenomic sequencing data prevents us from identifying the specific oral bacteria responsible for driving this pathway and clarifying the causal relationship between oral dysbiosis and the observed metabolic shifts. Therefore, future research integrating culturomics with metagenomic analyses is essential to isolate the relevant microorganisms and characterize the functional genes underlying this pathway's activation in periodontitis.

5 Conclusions

We have developed a novel method for collecting and analyzing salivary volatile metabolites by TD-GC-MS. This method holds significant promise for diagnosing periodontitis.

Our findings indicate that the diagnostic model, constructed from volatile metabolites such as cyclohexanone, ethanol, and styrene in saliva, demonstrates strong potential for periodontitis diagnosis. Furthermore, the caprolactam degradation pathway associated with cyclohexanone may play a crucial role in future studies investigating oral microbiota dysbiosis in periodontitis patients.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Local Ethics Committee of Peking University School of Stomatology and Stomatology Hospital (PKUSSIRB-202281149). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

QL: Data curation, Software, Supervision, Visualization, Writing – original draft. YZ: Conceptualization, Visualization, Writing – original draft. XZ: Conceptualization, Data curation, Software, Writing – review & editing. YLi: Data curation, Writing – review & editing. SX: Data curation, Visualization, Writing – review & editing. YLe: Software, Visualization, Writing – review & editing. XL: Supervision, Writing – review & editing. CY: Writing – review & editing.

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Conflict of interest

XZ, YL, XL were employed by Shimadzu (China) Co., LTD. China Innovation Center.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix 1: Analysis of saliva sample using LC-MS

Quality control sample

As a part of the system conditioning and quality control process, a pooled quality control sample (QC) was prepared by mixing equal volumes of all samples. The QC samples were disposed and tested in the same manner as the analytic samples. It helped to represent the whole sample set, which would be injected at regular intervals (every 5–15 samples) in order to monitor the stability of the analysis.

(UHPLC-MS/MS) analysis

The LC-MS/MS analysis of sample was conducted on a Thermo UHPLC-Q Exactive HF-X system equipped with an ACQUITY HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm; Waters, USA) at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The mobile phases consisted of 0.1% formic acid in water:acetonitrile (95:5, v/v) (solvent A) and 0.1% formic acid in acetonitrile:isopropanol:water (47.5:47.5, v/v) (solvent B). Positive ion mode separation gradient: 0–3 min, mobile phase B was increased from 0% to 20%; 3–4.5 min, mobile phase B was increased from 20% to 35%; 4.5–5 min, mobile phase B was increased from 35% to 100%; 5–6.3 min, mobile phase B was maintained at 100%; 6.3–6.4 min, mobile phase B was decreased from 100% to 0%; 6.4–8 min, mobile phase B was maintained at 0%. Separation gradient in negative ion mode: 0–1.5 min, mobile phase B rises from 0 to 5%; 1.5–2 min, mobile phase B rises from 5% to 10%; 2–4.5 min, mobile phase B rises from 10% to 30%; 4.5–5 min, mobile phase B rises from 30% to 100%; 5–6.3 min, mobile phase B linearly maintains 100%; 6.3–6.4 min, the mobile phase B decreased from 100% to 0%; 6.4–8 min, the mobile phase B was linearly maintained at 0%. The flow rate was 0.40 mL/min and the column temperature was 40°C.

MS conditions:

The mass spectrometric data were collected using a Thermo UHPLC-Q Exactive HF-X Mass Spectrometer equipped with an electrospray ionization (ESI) source operating in positive mode and negative mode. The optimal conditions were set as followed: source temperature at 425°C; sheath gas flow rate at 50 arb; Aux gas flow rate at 13 arb; ion-spray voltage floating (ISVF) at −3,500 V in negative mode and 3,500 V in positive mode, respectively; Normalized collision energy, 20–40–60 V rolling for MS/MS. Full MS resolution was 60,000, and MS/MS resolution was 7,500. Data acquisition was performed with the Data Dependent Acquisition (DDA) mode. The detection was carried out over a mass range of 70–1,050 m/z.

Analysis of the correlation between differential metabolites and age

TABLE A1 Analysis of the correlation between differential metabolites and age in the periodontitis group.

Metabolites	<i>R</i>	<i>R</i> ²	Adjusted <i>R</i> ²	<i>P</i>
2-Ethyl-oxetane	0.037	0.001	−0.008	0.708
Butanoic acid, methyl ester	0.063	0.004	−0.006	0.527
2-Pyrrolidinone, 1-methyl-	0.188	0.035	0.026	0.057
Ethanol, 2-butoxy-	0.033	0.001	−0.009	0.741
Ethanol	0.135	0.018	0.009	0.173
Cyclopentane, methyl-	0.031	0.001	−0.009	0.758
Decane	0.165	0.027	0.018	0.096
2,4-Dimethyl-1-heptene	0.237	0.056	0.047	0.016
Butylated Hydroxytoluene	0.090	0.008	−0.002	0.367
Undecane, 3-methyl-	0.055	0.003	−0.007	0.578
Cyclohexanone	0.071	0.005	−0.005	0.475
2,3-Butanedione	0.035	0.001	−0.009	0.725
Styrene	0.114	0.013	0.003	0.252
Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester	0.164	0.027	0.017	0.097

TABLE A2 Analysis of the correlation between differential metabolites and age in the healthy group.

Metabolites	<i>R</i>	<i>R</i> ²	Adjusted <i>R</i> ²	<i>P</i>
2-Ethyl-oxetane	0.733	0.537	0.515	<0.001
Butanoic acid, methyl ester	0.425	0.181	0.142	0.053
2-Pyrrolidinone, 1-methyl-	0.219	0.048	0.003	0.315
Ethanol, 2-butoxy-	0.140	0.019	−0.027	0.525
Ethanol	0.402	0.162	0.122	0.057
Cyclopentane, methyl-	0.715	0.512	0.489	<0.001
Decane	0.018	0.001	−0.470	0.935
2,4-Dimethyl-1-heptene	0.385	0.148	0.108	0.069
Butylated Hydroxytoluene	0.248	0.061	0.017	0.254
Undecane, 3-methyl-	0.305	0.093	0.050	0.157
Cyclohexanone	0.025	0.001	0.043	0.905
2,3-Butanedione	0.571	0.326	0.297	0.003
Styrene	0.145	0.021	−0.022	0.490
Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester	0.013	<0.001	−0.043	0.950

R: Strength and direction of the linear correlation between the independent and dependent variables.

R-squared (*R*²): Proportion of variance explained by the model.

Adjusted *R*-squared: *R*-squared adjusted for degrees of freedom, to prevent overfitting.

P-value: Tests whether the independent variable has a significant effect on the dependent variable.