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Age- and Periodontal Disease Independent Correlation of Salivary Amino Acids

SHOJI TANAKA[1], HIROSHI SAKAGAMI[1] and MASAHIRO SUGIMOTO[2,3]

[1]Meikai University School of Dentistry, Sakado, Japan;
[2]Institute for Advanced Biosciences, Keio University, Tsuruoka, Japan;
[3]Institute of Medical Science, Tokyo Medical University, Tokyo, Japan

Abstract

Background/Aim: This study explored the relationship between salivary metabolomic profiles, periodontal diseases, and age.
Patients and Methods: Resting whole saliva samples were collected from a cohort comprising 21 women and 30 men aged 20 to 70 years, including healthy volunteers and patients with different stages of periodontal diseases. Hydrophilic metabolites were analyzed using capillary electrophoresis-mass spectrometry. The concentrations were quantified and analyzed using multivariable analysis with or without normalization to eliminate overall differences in salivary concentrations across the samples.
Results: Metabolomic analysis quantified the absolute concentration of 248 metabolites in saliva samples. The unnormalized metabolomic profiles formed large clusters, with more than half of the detected metabolites showing positive correlations with each other. The absence of such clusters in the normalized data suggests the presence of individual differences in the processed data. The presence of urea, whose concentration increased gradually with the degree of progression of periodontal disease, and leucine, whose concentration decreased gradually, was identified. Highly positive correlations were observed between proline and glycine, which remained consistent regardless of normalization, age, or disease progression.
Conclusion: The metabolomic profiles of salivary samples revealed unique correlations between amino acids that were independent of age and periodontal disease.

Keywords: Saliva, aging, oral cavity, metabolomics, periodontal disease, amino acids.

Introduction

Human saliva, an intricate and multifaceted biological fluid, intricately amalgamates secretions from salivary glands with gingival crevicular fluid. This fluid plays a pivotal role in maintaining homeostasis within the oral cavity by executing a diverse array of functions, including oral digestion, preservation of tooth integrity, and providing antibacterial

Masahiro Sugimoto, Institute for Advanced Biosciences, Keio University, 246-2 Mizukami, Kakuganji, Tsuruoka, Yamagata, 997-0052, Japan. Tel: +81 235290528, Fax: +81 235290574, e-mail: msugi@sfc.keio.ac.jp

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and antiviral protection. Saliva can also reflect the systemic physiological state, making it a valuable medium for biomarker discovery to detect various diseases (1).

Saliva undergoes alterations due to diverse physiological processes within the oral cavity, which exhibit variations in tandem with aging. The decline in salivary flow rate associated with aging and modifications in the composition of antimicrobial and other proteins implies a potential connection to the age-associated dysfunction of specific glands (2) and the increase of diseases such as hypertension and diabetes (3). The diminution of both immunologic and non-immunologic defenses with aging may render oral soft tissues more susceptible to environmental influences, thereby inducing mucosal inflammatory responses (3). Consequently, elucidating the correlation between aging and the constituents of salivary composition holds clinical significance.

Metabolomics is an omics technology for comprehensively analyzing small molecules and understanding their relationship with phenotypes. This method enables the simultaneous quantification of hundreds of metabolites. In particular, non-targeted mass spectrometry (MS)-based analysis, which does not require the predefinition of the target to be measured, increases the potential to discover novel molecular associations. The relationship between salivary metabolomic profile and oral pathologies (4), neurodegenerative diseases (5), and cancers (6) has been studied.

The relationship between aging and metabolomic profiles has attracted attention (7-9). For example, both lipids and amino acids in plasma exhibit age-dependent changes (10). More than half of the plasma metabolites, particularly those involved in sphingolipid metabolism, were found to vary depending on age and sex (11). Age-related metabolomic changes in saliva are expected to be more complex, influenced by the functions mentioned above and many functional changes in the oral cavity, such as up-regulation of chronic inflammation, changes in oral bacteria, and decreased salivary secretion (12-14). Although salivary amino acids have been well-profiled for individual parameters such as caries (15, 16), periodontal disease (17, 18), cancers (19), and aging (20), integrated analysis of amino acids and other metabolites should be conducted to obtain the holistic view of the salivary characteristics.

In this study, we profiled hydrophilic metabolites in saliva collected from subjects with a wide range of ages and stages of periodontal diseases to investigate the association between salivary metabolites and health conditions in the oral cavity.

Patients and Methods

Subjects and saliva collection. This study was approved by the Ethics Committee of our University (protocol number A1113) on January 1, 2012. All participants, including patients and volunteers, provided written informed consent before providing saliva samples. Recruitment of volunteers took place from the patient population at our University Hospital.

Saliva was collected between 9:00 AM to 3:00 PM. To ensure the reliability of the samples, participants refrained from engaging in strenuous physical activity, consuming any substances aside from water, brushing their teeth, and performing oral care activities, such as using toothpicks one hour before saliva collection.

According to the experimental protocol, subjects rinsed their mouths with water, followed by a waiting period of 5 min. Subsequently, participants spat out resting whole saliva into 50 ml polypropylene tubes placed on a cup containing crushed ice. Participants were well-instructed not to spit out mucus during the collection process. The collected saliva samples (0.5 to 1.0 ml) was promptly frozen at −80˚C for subsequent analysis.

The degree of periodontal disease was determined based on clinical parameters evaluated through intraoral examination and radiographs, including the degree of gingivitis, bone resorption, probing depth, bleeding on probing, and tooth movement. Patients were classified into four groups, based on the periodontal index (PI) (21): control (healthy control subject) (C) (PI <1.0), low (L) (PI=1.0-3.0), moderate (M) (PI=3.1-5.0), and high degree of PI (H) (PI=5.1-8.0).

Metabolomic analysis. Saliva was thawed and passed through a 5 kDa-cutoff filter (Millipore, Burlington, MA, USA) with centrifugation at 9,100×*g* for at least 2.5 h at 4˚C to remove macromolecules. Five microliters of Milli-Q water containing internal standards (2 mmol/l each of methionine sulfone, 2-[*N*-morpholino]-ethanesulfonic acid, D-camphor-10-sulfonic acid, 3-aminopyrrolidine, and trimesate) was added to 45 μl of the filtrate and mixed immediately before capillary electrophoresis time-of-flight-mass spectrometry (CE-TOFMS) analysis. The parameters and instruments of CE-TOFMS are described elsewhere (22).

Data analysis. Raw data were analyzed using MasterHands, which detected all possible peaks, eliminated noise and redundant features, to generate the aligned data matrix (23). Metabolite identification was conducted by matching the *m/z* values and the normalized migration times with those of our standard compound libraries. The peak area of each metabolite was integrated and divided by one of the internal standards to yield a relative peak area. After confirming the measurement is done under the linearity ranges, the absolute concentrations of samples were calculated based on the peak area. The peaks smaller than lower quantification limits were treated as not detected peaks. All samples were measured in a single batch. The order of the sample sequence was randomized to eliminate unexpected bias.

A heatmap with clustering Elucidation distance and Ward methods were used to visualize a full view of the metabolites. Principal component analyses were also used to score and load plots. The correlation between metabolites and age was also analyzed using the Spearman correlation. All analyses were conducted using absolute concentrations and normalized ones. Here, we used quantile normalization to match the quantiles of the data among samples.

These analyses were conducted using MetaboAnalyst (ver. 6.0, https://www.metaboanalyst.ca/) and GraphPad Prism (ver. 9.5.1, Intuitive Software for Science, San Diego, CA, USA).

Table I. *Subject characteristics.*

	Total	C	L	M	H
Sex					
Female	21	3	5	7	6
Male	30	5	9	8	8
Age					
20s	7	7	0	0	0
30s	3	1	2	0	0
40s	3	0	1	1	1
50s	10	0	3	3	4
60s	22	0	7	9	6
70s	6	0	1	2	3

C indicates healthy controls. L, M, and H indicate the low, middle, and high progression of periodontal diseases.

Results

Table I shows subject characteristics. Metabolomics analysis identified 248 types of small molecules, with 114±15 [mean±standard deviation (SD)] of metabolites in each saliva sample. Overall hydrophilic metabolites in primary metabolic pathways, such as glycolysis, tricarboxylic acid cycle, urea cycle, nucleotide, and amino acids, were quantified.

Figure 1 depicts the overview of the profiled concentrations of metabolites of saliva. The profiles of healthy controls (C) and periodontal diseases with low (L), middle (M), and high progression (M) were compared. Figure 1A presents the results using absolute concentrations, and Figure 1B shows the results with concentrations normalized between samples to match their quartiles. The heatmap (Figure 1A) includes a large cluster in the bottom half of the metabolites between ornithine and valine (Val), indicating higher concentrations of these metabolites in the C group compared to those in the other groups. The heatmap with normalized data (Figure 1B) includes a smaller cluster in the C group between gamma-butyrobetaine and 2-aminobutyric acid (2AB). Instead, large clusters appear in the other groups, such as between urocanate and alanine-alanine (Ala-Ala) in the L group and between beta-alanine (beta-Ala) and hypoxanthine in the M group.

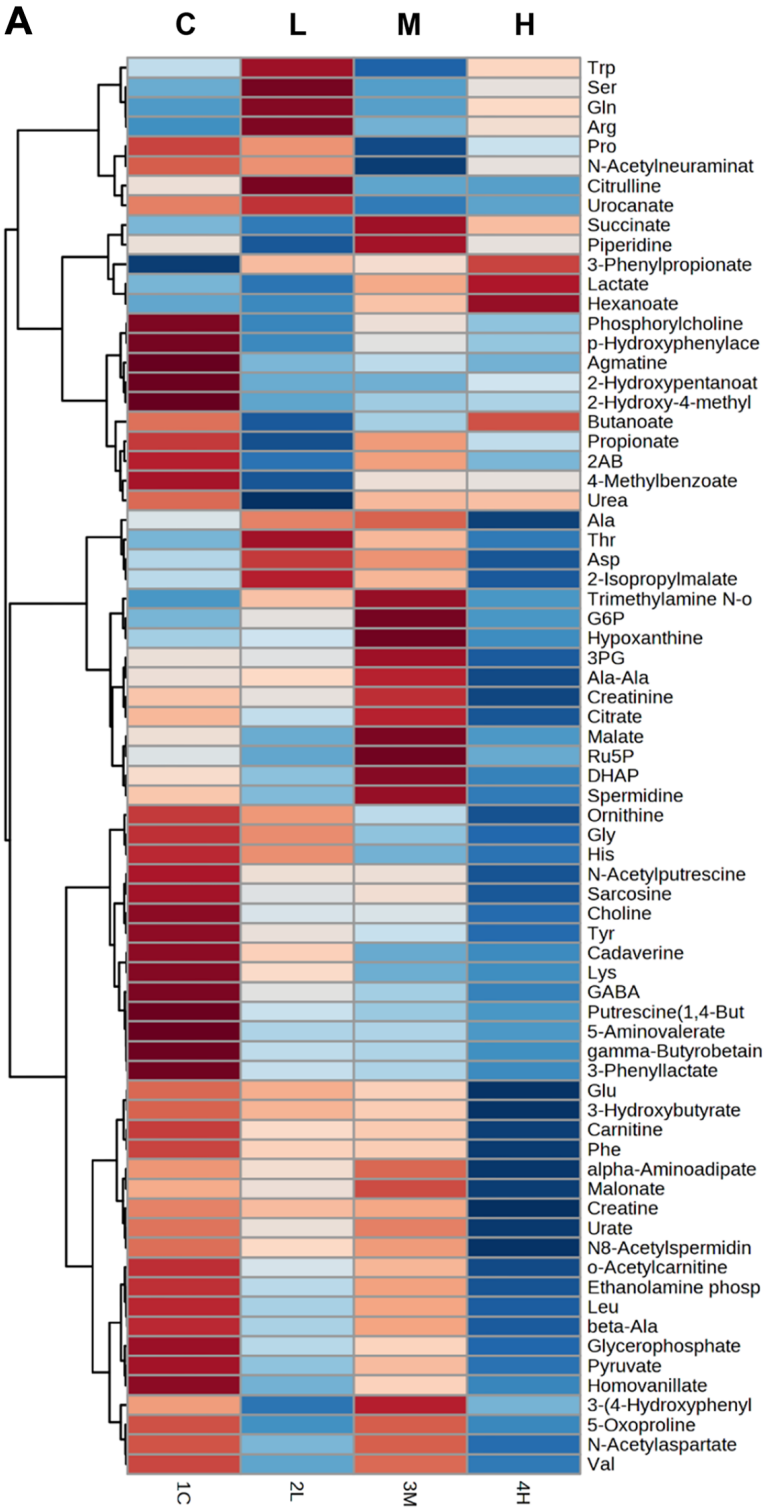

Figure 1. *Continued*

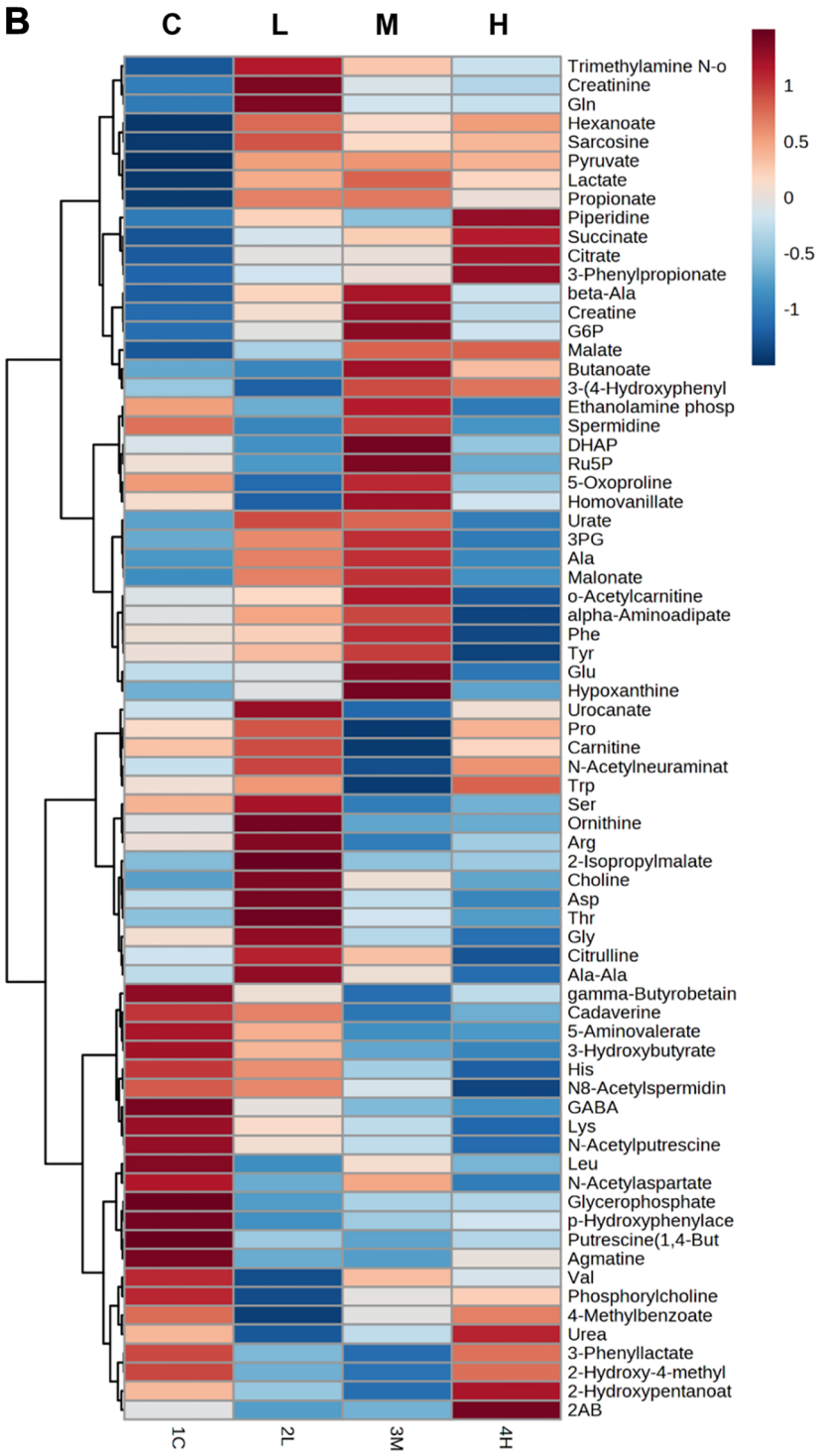

Figure 1. *Heatmap of salivary metabolomic profiles. A) Data without normalization. B) Data with normalization. Healthy controls (C) and periodontal diseases with low (L), middle (M), and high (H) progress. The average values of each group are visualized. The value of each metabolite is transferred into Z-scored, with red and blue indicating relatively high and low values, respectively. The average value is shown in white.*

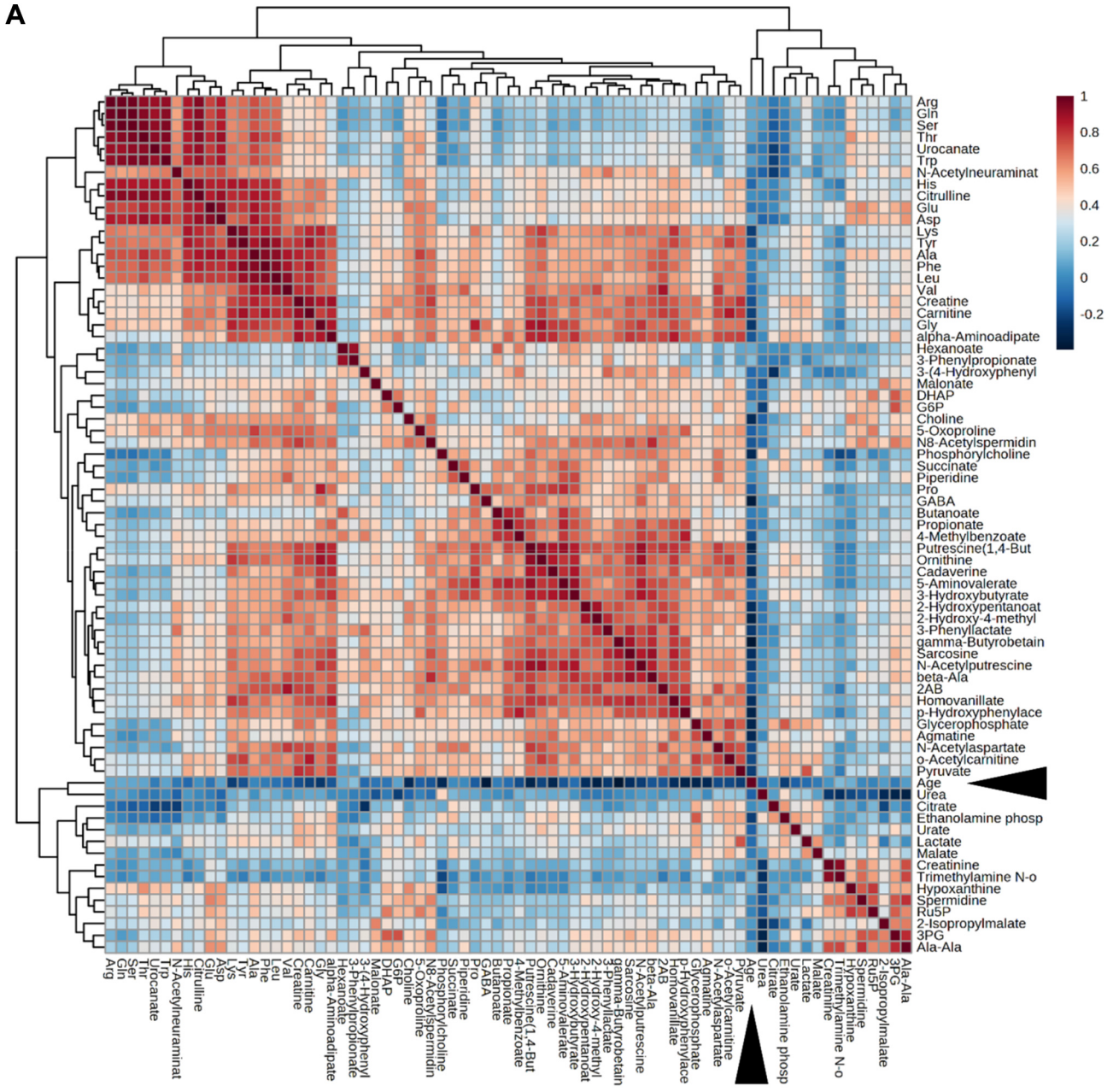

Figure 2. *Continued*

Figure 2 depicts the correlations between all profiled metabolites and age. Figure 2A presents the analysis results using absolute concentrations, whereas Figure 2B shows the results using normalized data. The analysis of absolute concentrations yields larger clusters, colored red, that include metabolite concentrations that are positively correlated (Figure 2A). After normalization, Uroconate, serine (Ser), and threonine (Thr) exhibit high positive correlations in the upper left of the heat map (Figure 2B). Glutamine (Gln) and tryptophan (Trp) also exhibit highly positive correlations, but

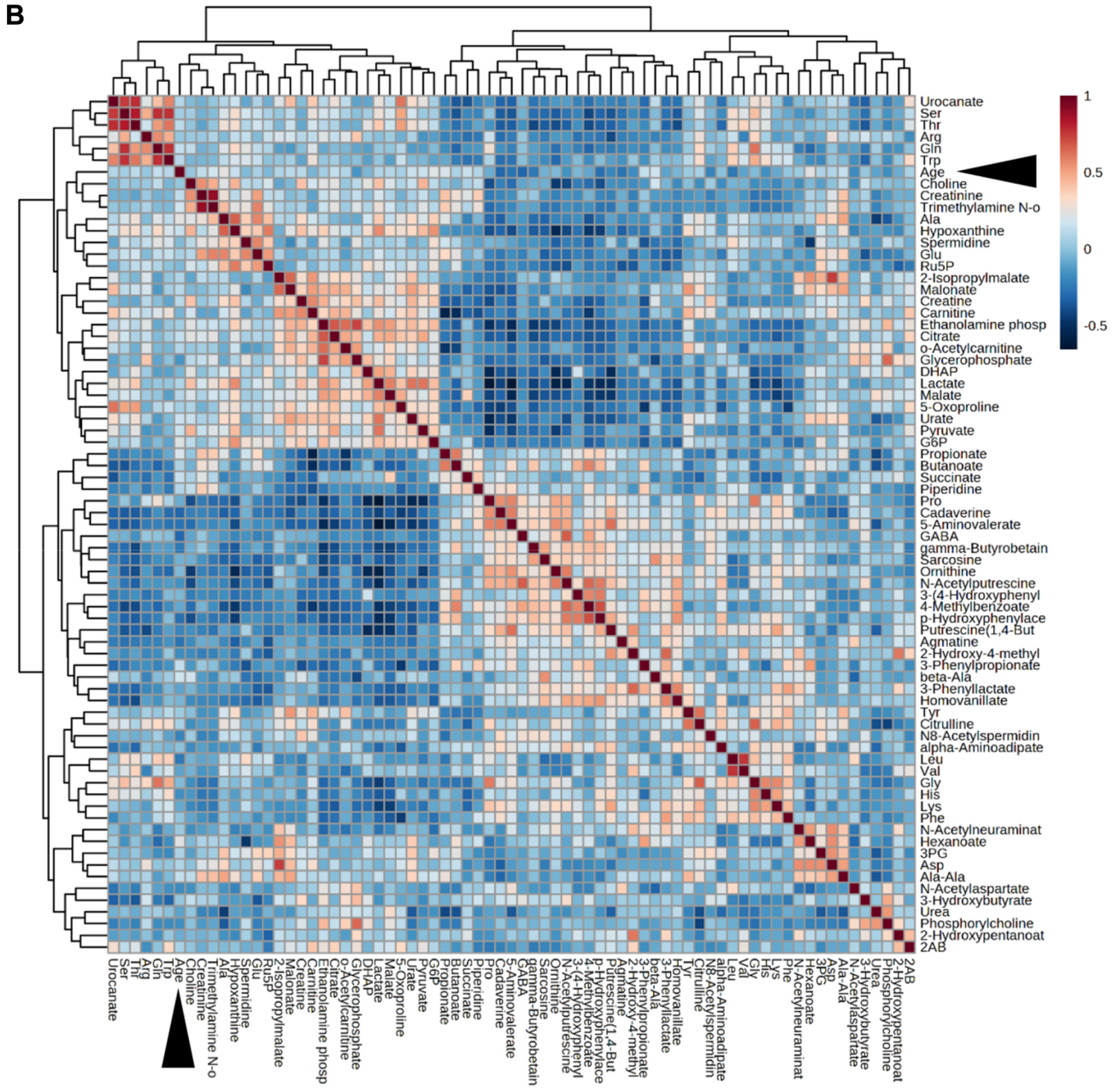

Figure 2. *Heatmap of correlation of metabolites and age. A) Data without normalization. B) Data with normalization. Spearman correlation (R) was used for the analysis. Red and blue indicate positive and negative correlations, respectively. Black triangles indicate the data of age.*

the cluster sizes are smaller (Figure 2B). The clusters for the other metabolites show weaker positive correlations than when absolute concentrations were used. No metabolites showed a high positive correlation with age.

Figure 3 depicts the results of the principal component analysis. When absolute concentrations are utilized, score plots (Figure 3A) and loading plots (Figure 3B) are presented. The analysis results with normalization are

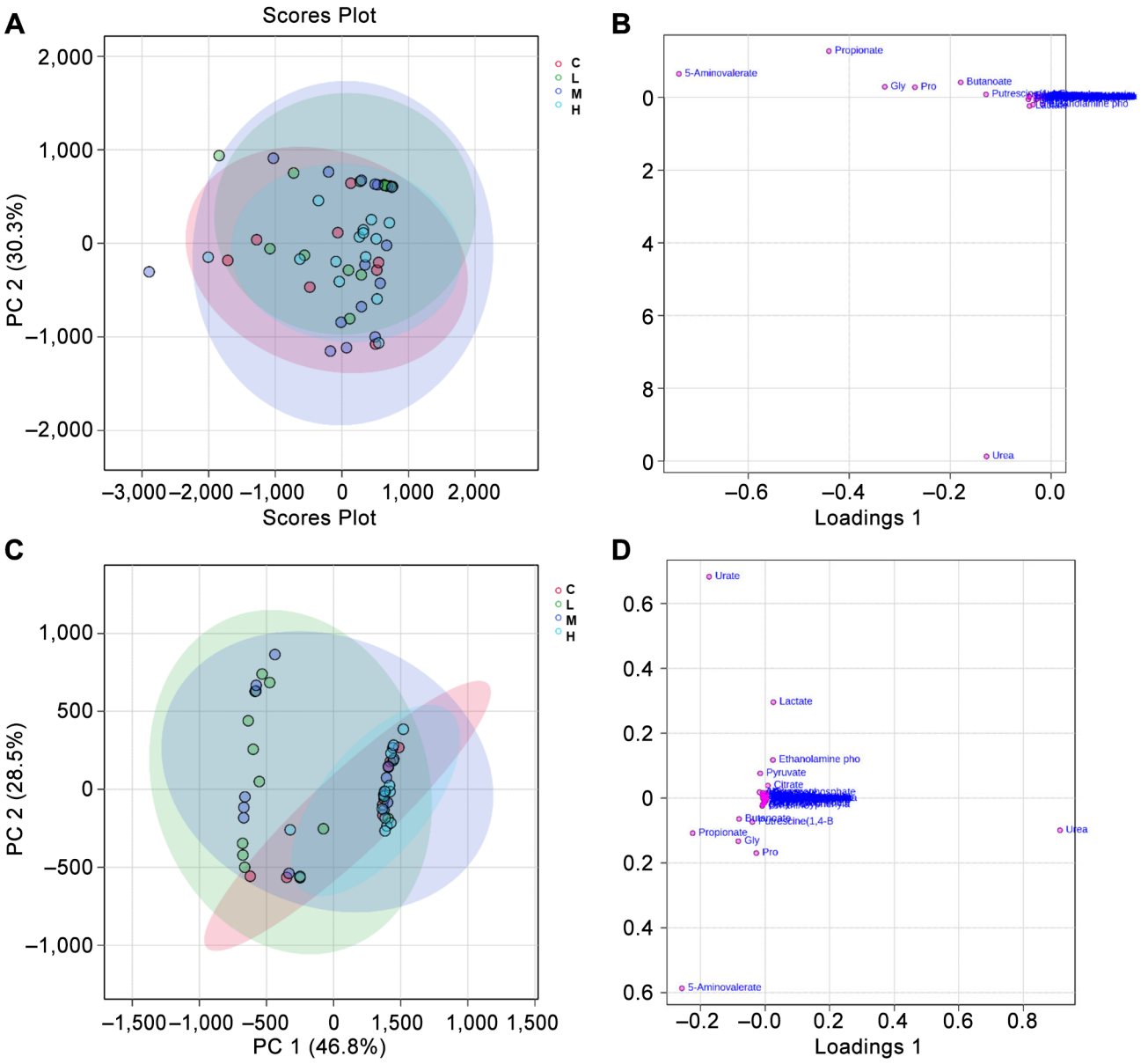

Figure 3. *Results of principal component analysis. A, B) Data without normalization. C, D) Data with normalization. A, C) Score plots. A plot indicates a sample. B, D) Loading plots. A plot indicated a metabolite. Healthy controls (C) and periodontal diseases with low (L), middle (M), and high (H) degree.*

depicted in Figure 3C and D. Each score plot represents a sample, and proximity between plots indicates a high similarity of metabolic concentrations in these samples. Figure 3A shows score plots using absolute concentrations, which reveal overlap in four groups. The loading plots indicate that urea has a pattern distinct from the other metabolites and is located at the bottom of the figure. Similarly, 5-aminovalerate, propionate, glycine (Gly), and proline (Pro) are also positioned at the bottom of the X-axis. All metabolites located far from most others had negative values on the X-axis. Only urea had a negative value on the Y-axis, while the other metabolites had positive values.

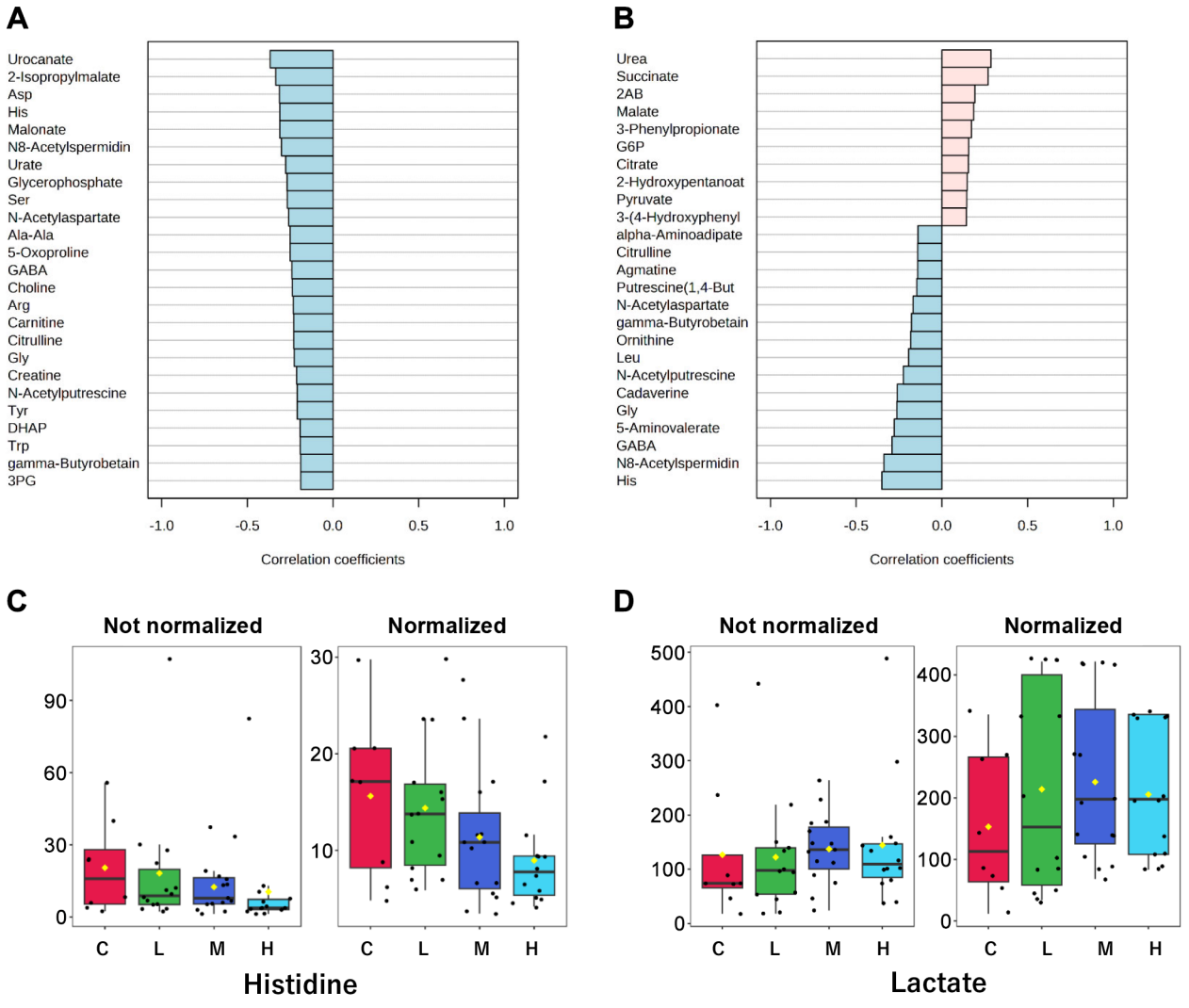

Figure 4. *The results of PatternHunter-based analysis. A) Data without normalization. B) Data with normalization. Metabolites showed a tendency to increase and decrease in the order of C-L-M-H, corresponding to positive and negative values, respectively. Box plots of C) histidine and D) lactate.*

The score plots using normalized data (Figure 3C) indicate that most samples are clustered on the right side, while some samples in the L and M groups are scattered on the left. In the loading plots (Figure 3D), most metabolites clustered near X=0 and Y=0. The plot of urea is located on the right with a large X value. Urate, propionate, and 5-aminovalerate showed negative values outside the X-axis. Lactate and pyruvate, the end products of glycolysis, also had positive X values. Two amino acids, glycine (Gly) and proline (Pro), are located in the lower left area.

Figure 4 shows the results of the correlation of metabolites with the progression of periodontal disease. Metabolites with increasing or decreasing values in the order C-L-M-H were analyzed using the Pattern Hunter function of MetaboAnalyst. The top 25 metabolites with these trends are shown. Without normalization, all metabolites tended to decrease in the order C-L-M-H (Figure 4A). With normalization, ten metabolites, such as urea and succinate, tended to increase, while 15 metabolites tended to decrease (Figure 4B). For instance, histidine

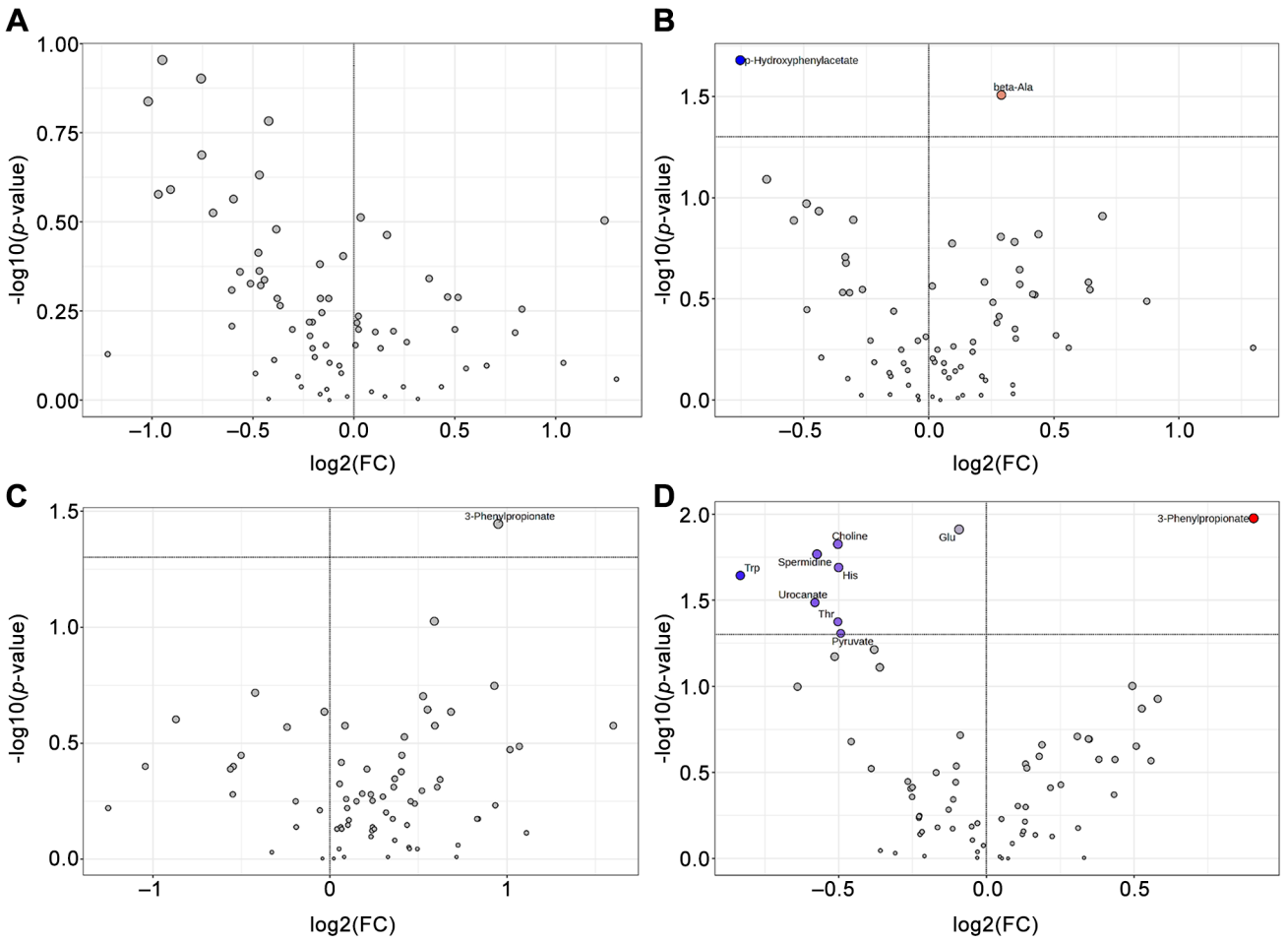

Figure 5. *Volcano plots showing the effects of age and sex. A, B) Age. C, D) Sex. A, C) Data without normalization. B, D) Data with normalization. A, B) X-axis indicates* $log_2(Y/E)$*. C, D) X-axis indicates* $log_2(W/M)$*. Y-axis indicates* $-log_{10}(P)$*. p-value is calculated by Mann-Whitney test. Y>1.3 equals p<0.05. The metabolite above the horizontal bars shows statistical significance.*

exhibited a decreasing trend before normalization, which further decreased after normalization (Figure 4C). Lactate showed no significant change before normalization; however, this metabolite showed an increasing trend after normalization (Figure 4D).

Figure 5 shows the impact of age and sex on the salivary metabolic profiles. The data were divided into two groups by the median age of 61 years: younger (Y) and elder groups (E). Figure 5A does not show any significantly different metabolites without normalization. Figure 5B, however, displays significant differences in beta-Ala and p-hydroxyphenylacetate after normalization (Mann-Whitney *U*-test). Considering sex, the data was divided into two groups: female (W) and male (M). Figure 5C shows the results without normalization, indicating that only 3-phenylpropionate was significantly higher in females. Figure 5D presents the results with normalization, revealing that 3-phenylpropionate was significantly higher in females, and eight metabolites, including amino acids, were significantly higher in males. The two-group comparison between Y and E was inconsistent depending on the use of normalization. The two-group comparison of sex was consistent only for 3-phenylpropionate.

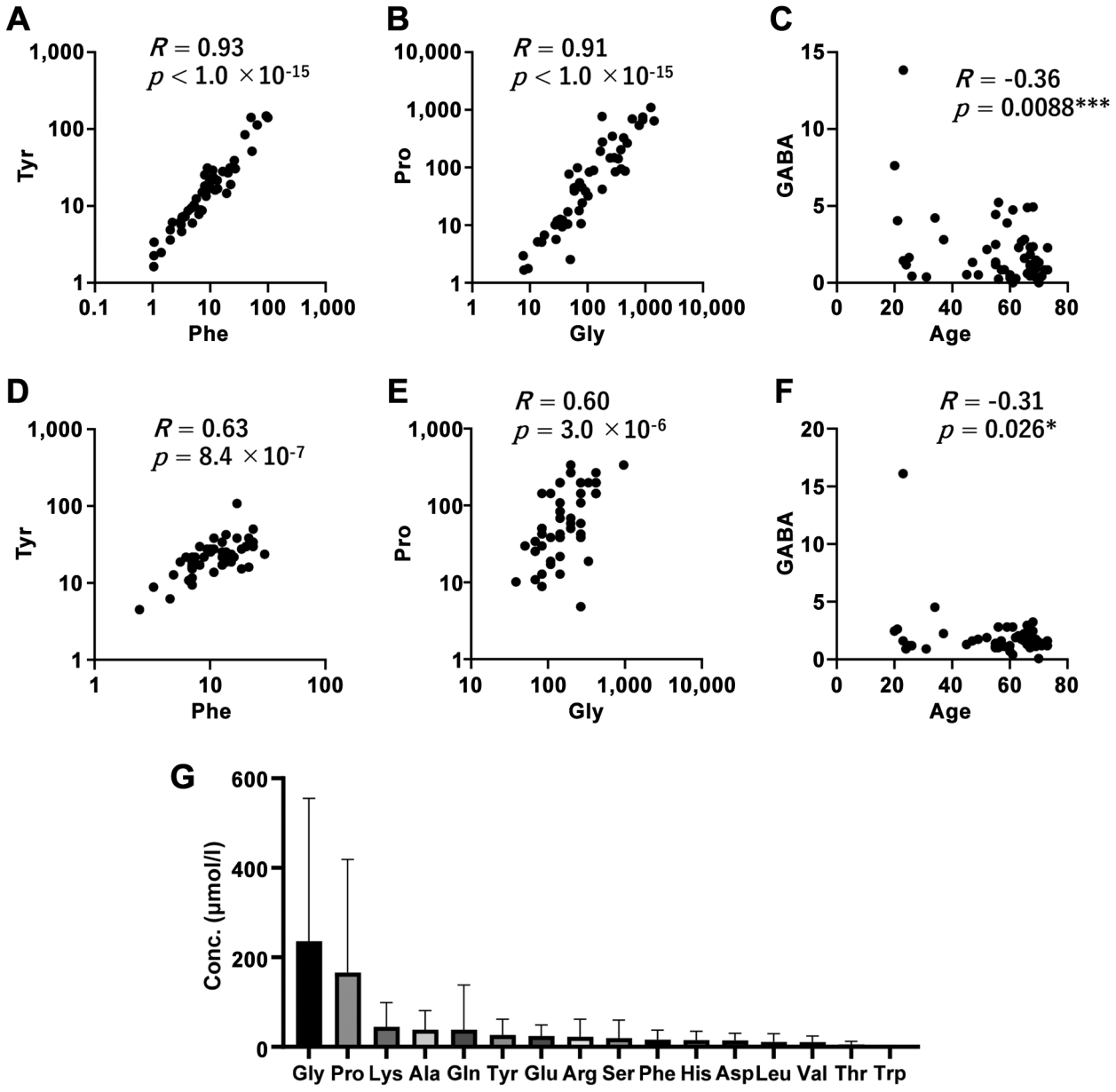

Figure 6. *Correlation between a metabolite and age. A, B, C) Data without normalization. Unit of both axes is mmol/l. D, E, F) Data with normalization. A, D) Tyrosine (Tyr) and Phenylalanine (Phe). B, E) Glycine (Gly) and Proline (Pro). C, F) Age and GABA. Spearman correlation (R) was used for the analysis. G) The average and standard deviation of the absolute concentrations of amino acids.*

Figure 6 shows the correlations between amino acids and age. The highest correlation among the amino acids was found between phenylalanine (Phe) and threonine (Thr), with R=0.93 (p<1.0×10^{-15}) without normalization (Figure 6A) and R=0.63 (p<8.4×10^{-7}) with normalization (Figure 6D). Principal component analysis revealed that Gly and Pro exhibited distinct behavior from other amino acids in the loading plots in analyzing various degrees of periodontal diseases. Their concentrations showed a significant positive correlation of R=0.91 (p<1.0×10^{-15})

and R=0.60 ($p<3.0\times10^{-6}$) without/with normalization (Figure 6D). The metabolite showing the highest absolute correlation coefficient with age was GABA. However, before normalization (Figure 6C) and after normalization (Figure 6F), it exhibited a negative correlation coefficient, suggesting that its value tends to decrease with age.

The amino acids with the highest concentration in saliva were glycine and proline, followed by lysine and alanine. The concentrations of proline and glycine were found to be linearly related, with proline being 0.684 times the glycine concentration, which means that the glycine/proline ratio was 1.46.

Discussion

This study analyzed the effect of periodontal disease on saliva metabolites in normal volunteers and patients with periodontal disease at different ages. Metabolomic analysis was used to examine the magnitude of the effect. The multivariate analysis showed different results for absolute concentrations of metabolites in saliva, and relative concentrations were determined by normalization between samples. In the case of metabolite relationships, many substances showed correlations when normalization was not performed, indicating the influence of overall shading between saliva samples (Figure 2A). However, multiple highly correlated clusters revealed a complex pattern of profiles rather than a simple influence on overall shading (Figure 2B). When normalizing to match quartiles across samples, several small clusters could be observed instead of the large correlative clusters containing many metabolites. These observations indicate that some metabolite groups fluctuate relative to the overall shading (Figure 2B).

In the loading plots of the principal component analysis related to periodontal disease, most metabolites clustered at 0 points. However, 5-aminovalerate and propionate showed positions outside of 0 points without normalization. After normalization, urate, urea, lactate, and others showed positions off from 0. Urate is the primary antioxidant in saliva, and its activity is reduced in patients with periodontal disease (24). Lactate is produced from pyruvate by lactate dehydrogenase. Decreased concentrations of lactate have been reported in the saliva of periodontal patients (25). Our data showed that the absolute concentration of lactic acid did not fluctuate significantly with age but increased with age after normalization. Using the concentration of a single substance alone as a marker of disease is difficult due to variations with age and normalization. Therefore, it is necessary to analyze changes in the patterns of multiple substances to diagnose a disease accurately.

Regarding saliva components, there have been several biochemical comparisons with blood components and analytical studies of the saliva metabolome. However, the reported results were contradictory (26). It is important to note that while absolute concentrations are used for blood components, results may vary in saliva depending on normalization. Therefore, multifaceted analysis is necessary before concluding. It is widely recognized that the biochemical function of salivary glands declines with age (27, 28). Studies on animals have reported decreased protein synthesis function in the rat parotid gland, which is not dependent on reducing cell number or cellular metabolism (29). However, a significant variation in metabolites in saliva based on oral health and age was also found (30). Younger and middle-aged individuals showed more significant variation due to periodontal disease, while older individuals showed more significant variation due to tooth loss (30).

Regarding salivary metabolites, several substances were decreased in older individuals compared to younger ones, except for ATP. Additionally, many of the detectable metabolites were found to be correlated with each other (13). In our data, only a few substances correlated with age, and the correlations between substances varied depending on whether they were normalized or not (Figure 2 and Figure 5). In disease marker discovery, many investigators have tried to design an age-matched study (14). The question is how to normalize the data when including various age groups in the analysis.

However, amino acids with and without normalization exhibited significantly positive correlations (Figure 6). Tyr and Phe displayed the strongest correlation among the amino acids, while Gly and Phe exhibited different variability than the other amino acids in relation to periodontal disease, and showed significantly different positive correlations. The ratio of Gly/Pro, which had the highest concentration of amino acids, was 1.46.

The ratio of Gly/Pro in collagen is 2.94 (31). However, the Gly/Pro ratio in saliva does not agree with this, suggesting that collagen breakdown products are not the major source of amino acids. This correlation is strong, despite the inclusion of data for various ages, degrees of periodontal disease, and both sexes. Further investigation is needed to understand the circumstances under which these disruptions occur.

Study limitations. First, CE-MS was only used for water-soluble metabolites, not fat-soluble components. Secondly, the normalization method used between samples was based on matching quartiles of observed metabolite concentrations, which depends on the available data. This method cannot be used for general purposes unless it normalizes a single molecule, such as creatinine in urine. However, the metabolite profiles observed in this study exhibited patterns that were not simply general shading. Therefore, normalization would require the use of multiple molecules. Additionally, confirming the correlation of the observed amino acids in this study with independent data is crucial.

Conclusion

Salivary metabolomic profiles were analyzed using capillary electrophoresis-mass spectrometry. Multivariable analysis showed inconsistent results depending on the use of normalization. The highly positive correlations of several amino acids were observed independently on the normalization. These correlations were independent of age and the progress of periodontal diseases. This distinctive amino acid pattern may indicate a homeostasis of the oral cavity.

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Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

Shoji Tanaka: Investigation, Funding acquisition, Data curation, Methodology, Writing - review & editing. Hiroshi Sakagami: Conceptualization, Project administration, Writing - review & editing. Masahiro Sugimoto: Formal analysis, Methodology, Original draft, Writing - review & editing.

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