Analysis of salivary phenotypes of generalized aggressive and chronic periodontitis through nuclear magnetic resonance-based metabolomics

Federica Romano[*], Gaia Meoni[†‡], Valeria Manavella[*], Giacomo Baima[*], Leonardo Tenori[†‡], Stefano Cacciatore[§], Mario Aimetti[*]

[*]Department of Surgical Sciences, C.I.R. Dental School, University of Turin, Turin, Italy.

[†]Magnetic Resonance Center (CERM), University of Florence, Sesto Fiorentino, Italy.

[‡]Department of Experimental and Clinical Medicine, University of Florence, Sesto Fiorentino, Italy.

[§]Department of Surgery & Cancer, Imperial College, London, UK and International Centre for Genetic Engineering and Biotechnology, Cancer Genomics Group, Cape Town, South Africa.

Federica Romano and Gaia Meoni contributed equally to this article.

Corresponding author:

Prof. Mario Aimetti, Section of Periodontology, C.I.R. Dental School, Via Nizza 230 10126 Turin (Italy). Phone: +390116331543 email: mario.aimetti@unito.it Fax: +390116331506 (Fax number and e-mail can be published).

Word count: 3460

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/JPER.18-0097.

Number of Tables: 2

Number of Figures: 4

Number of references: 46

Running title: Salivary metabolomics in periodontitis

One sentence summary: Metabolomic analysis of saliva discriminates healthy individuals from periodontitis patients, irrespectively of the aggressive or chronic periodontitis profile.

Abstract

Background: Recent findings about the differential gene expression signature of periodontal lesions have raised the hypothesis of distinctive biological phenotypes expressed by generalized chronic periodontitis (GCP) and generalized aggressive periodontitis (GAgP) patients. Therefore, this cross-sectional investigation was planned, primarily, to determine the ability of nuclear magnetic resonance (NMR) spectroscopic analysis of unstimulated whole saliva to discriminate GCP and GAgP disease-specific metabolomic fingerprint and, secondarily, to assess potential metabolites discriminating periodontitis patients from periodontally healthy individuals (HI).

Methods: NMR-metabolomics spectra were acquired from salivary samples of patients with a clinical diagnosis of GCP (n = 33) or GAgP (n = 28) and from HI (n = 39). The clustering of HI, GCP and GAgP patients was achieved by using a combination of the Principal Component Analysis and Canonical Correlation Analysis on the NMR profiles. **Results:** These analyses revealed a significant predictive accuracy discriminating HI from GCP, and discriminating HI from GAgP patients (both 81%). In contrast, the GAgP and GCP saliva samples seem to belong to the same metabolic space (60% predictive accuracy). Significantly lower levels ($P < 0.05$) of pyruvate, *N*-acetyl groups and

lactate and higher levels ($P < 0.05$) of proline, phenylalanine, and tyrosine were found in GCP and GAgP patients compared with HI.

Conclusions: Within the limitations of this study, CGP and GAgP metabolomic profiles were not unequivocally discriminated through a NMR-based spectroscopic analysis of saliva.

Keywords: biomarkers, metabolomics, magnetic resonance spectroscopy, periodontal diseases, saliva.

Introduction

Periodontitis is a multifactorial, chronic, inflammatory disease that leads to loss of periodontal attachment to the root surface and alveolar bone resorption and, if untreated, ultimately results in tooth exfoliation.[1] It is widely accepted that dysbiosis within the human dental plaque biofilm is the primary initiator of periodontitis[2], even though the extent and severity of tissue destruction appear to be host-mediated.[3,4]

Periodontitis can have heterogeneous clinical presentations. The traditional classification recognizes two major forms of periodontitis, chronic periodontitis (CP) and aggressive periodontitis (AgP), differing in rate of progression, prognosis and need for specific treatment approaches.[5] At the present, the diagnosis AgP and CP is primarily based on clinical examination and radiographic parameters.[5] No clinical, histopathological or microbiological assessment provides an unequivocal discrimination between the two conditions.[6,7] For this reason, there is a strong effort to discover specific molecular arrays as a diagnostic tool to differentiate CP and AgP by oral-health professionals.[8]

Several molecules in the oral fluids, namely gingival crevicular fluid (GCF) and saliva, have been investigated so far in the attempt to provide detailed understanding of the biochemical network of periodontal tissue destruction.[9] Saliva is particularly promising as it contains locally produced

proteins, as well as other molecules from the systemic circulation.[8] Furthermore, its collection is non-invasive, rapid and inexpensive.[10] Salivary diagnostics has already proved efficient in identifying alterations in oral and systemic health status.[11,12] Even so, various challenges persist regarding the use of saliva as a medium for an accurate and cost-effective detection of periodontitis, mainly due to the lack of specific markers of disease.[13]

Metabolomics is a newly emerging field of research dealing with the high-throughput identification and quantification of the whole ensemble of metabolites (small molecules; <1500 Da) in a cell, tissue, body fluids or ecological systems.[14] The metabolomics profiling reflects the dynamic response of a living system to genetic modification and physiological, pathological, and developmental stimuli.[15] Thus, metabolomics offers the potential for a holistic approach to an individualized, patient-centered medicine.

Compared with other high throughput approaches, the main benefit of metabolomics analysis resides in its ability to take a snap at the very end-point of all the complex causal pathways driving periodontal pathogenesis. Small molecules derived from the dysbiotic community and host tissue breakdown, targeted by metabolomics, are potentially able to reflect the real-time molecular phenotype of the disease.[9] At the same time, it has been proven that saliva is a stable biofluid and that a clear individual metabolic phenotype can be revealed using saliva samples.[16]

Untargeted metabolomics by nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) has been previously employed to differentiate healthy and periodontally diseased individuals through the pattern recognition analysis of saliva and GCF, since this approach has the advantage to maximize the number of metabolites detected, including chemical unknowns.[17-19] Although some studies provided promising preliminary outcomes regarding the detection of some panels of discriminant metabolites, further trials with larger sample sizes are needed in order to add consistency and external validity to these results.[17-20]

To the best of our knowledge, the possibility to employ the NMR-based metabolomics analysis to discriminate CP and AgP remains to explore. This pilot study was designed to test the hypothesis that untargeted metabolomic analysis of saliva could differentiate the biochemical signatures of the generalized forms of chronic periodontitis (GCP) and aggressive periodontitis (GAgP). The secondary aim was related to the detection of a differentially expressed array of metabolites that could be further investigated as potential biomarkers for the development of a rapid diagnostic tool for periodontitis.

Material and Methods

The protocol of this cross-sectional study was approved by the Institutional Ethical Review Board (protocol number 1503/2016) and the study was conducted in accordance with the Helsinki Declaration. Written informed consent was obtained from all participants. The study was reported according to the STROBE guidelines.21.

Study population

The sample size was set at 100 individuals based on the results of previous studies[19,22] and the pilot nature of this study. A total of 33 patients with GCP (mean age: 50.5 ± 8.9 years, 63.6% males and 15.2% smokers), 28 patients with GAgP (mean age: 31.1 ± 4.6 years, 64.3% males and 14.3% smokers) and 39 periodontally healthy individuals (mean age: 46.6 ± 8.2 years, 64.1% males and 15.4% smokers) were consecutively recruited from among individual seeking oral health consultation at the C.I.R. Dental School, University of Turin (Italy) from January to September 2017. After being screened, participants were balanced with respect to gender, and smoking habits.

Exclusion criteria included less than 20 teeth; antibiotic intake within the previous 3 months, periodontal treatment during the past 6 months, abnormal salivary function, diagnosis of any disease in oral and hard tissues and other systemic conditions that could influence periodontal status and

metabolomic profile (e.g. diabetes mellitus and metabolic syndromes), regular alcohol consumption, pregnancy and lactation.[23]

Determination of periodontal status and saliva collection

All participants underwent a periodontal examination by two experienced clinicians (V.M, F.R) who were previously trained and calibrated for the periodontal examination and saliva sampling. A set of full-mouth periapical radiographs was taken for each patient. Presence/absence of plaque (PI), presence/absence of bleeding on probing (BoP), probing depth (PD), and clinical attachment level (CAL) were measured at six sites around each tooth by manual probing[§ ||]. Inter-examiner reliability was determined by having each examiner made dual measurements along with those of the Project Director (M.A) on 15 non-study patients, and intra-examiner reproducibility was assessed by taking replicate measurements on the same patients with an interval of 24 hours between the first and the second recording. The percentage of agreement within 1 mm of PD and CAL ranged between 94% and 97%.

Patients with GCP and GAgP and healthy controls were diagnosed based on the current classification of the 1999 International Workshop for the Classification of Periodontal Diseases and Conditions[5] and met the following criteria. GCP patients had ≥ 30% of sites with PD and CAL > 5 mm, and presence of BoP.[5] Patients in the GAgP group were <35 years of age, and had at least six permanent first molars and/or incisors with at least one site with PD and CAL > 5 mm as well as a minimum of six teeth other than first molars and incisors also presenting at least one site each with PD and CAL > 5 mm.[24] Other factors such as family aggregation, rapid progression and the relationship between local factors and periodontal destruction were also considered.[5] The control group comprised healthy

[||] PCP UNC 15, Hu-Friedy, Chicago, IL.

individuals (HI) with PD and CAL ≤ 3 mm at all sites on all teeth, no radiographic evidence of alveolar bone loss, and <15% of sites presenting BoP.[24]

At least 24 hours after periodontal measurements to avoid blood contamination, unstimulated whole saliva was obtained by all study subjects between 8:00 and 10:00 am using standard techniques as described by Silwood et al.[25]. Briefly, all subjects were advised to refrain from using mouthwash and brushing their teeth at least 1 h before sample collection. Each subject was instructed not to force salivation, to allow saliva to be collected in the mouth, and let the saliva drain into a sterile graduated tube for 10 min. About 1 ml of saliva was collected from every patient and immediately frozen.

NMR sample preparation

Frozen saliva samples were thawed at room temperature and were centrifuged (5000 × *g* for a period of 30 min at 4°C) to remove debris. A total of 300 µl of sodium phosphate buffer (70 mM Na_2HPO_4; 20 % (v/v) ^2H_2O; 6.15 mM NaN_3; 6.64 mM sodium trimethylsilyl [2,2,3,3-$^2H_4$] propionate (TMSP); pH 7.4) was immediately added to 300 µl of each sample, and the mixture was homogenized by vortexing for 30 s. NaN_3 was added as a preservative to ensure that metabolites were not generated or consumed due to bacteria present in the saliva during the time of preparation of the samples or of the acquisition of NMR spectra. A total of 450 µl of this mixture was transferred into a 4.25 mm NMR tube[¶] for analysis.

NMR spectral acquisition

NMR spectra for all samples were acquired using a spectrometer[#] operating at 600.13 MHz proton Larmor frequency equipped with a 5 mm CPTCI ^{1}H-^{13}C-^{31}P and ^{2}H-decoupling cryoprobe including a z axis gradient coil, an automatic tuning-matching (ATM) and an automatic sample changer. A BTO

[¶] Bruker BioSpin srl, Milan, Italy.

[#] Bruker BioSpin srl, Milan, Italy.

2000 thermocouple served for temperature stabilization at the level of approximately 0.1 K at the sample. Before measurement, samples were kept for at least 3 min inside the NMR probehead, for temperature equilibration (300 K). For each saliva sample, a ^{1}H-NMR spectrum was acquired using the pulse sequence NOESY-presat with 64 free induction decay (FID) collected into 65536 data points over a spectral width of 12019 Hz, relaxation delay (RD) of 4 s and mixing time of 0.1s.

Spectral processing and analysis

Free induction decays were multiplied by an exponential function equivalent to a 1.0 Hz line-broadening factor before applying Fourier transform. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated using a RMN processing software[**]. Spectra were aligned by calibrating the TMSP peak at 0.00 ppm. Each 1D spectrum in the spectral ranges 0.2−4.3 and 6.6−10.0 ppm was segmented into 0.02 ppm chemical shift bins, and the corresponding spectral areas were integrated using a specific software program††. The binning procedure is a mean to reduce the number of total variables, to compensate for subtle signal shifts, and filter noise in the spectra, making the analysis more robust and reproducible.[26,27] The total spectral area was calculated on the bins and total area normalization was carried out on the data prior to pattern recognition.

Statistical analysis

All data analyses were performed blindly using R statistical package. Significance difference among the clinical groups was calculated using analysis of variance for clinical data and post hoc significance of differences between pairs of comparisons was determined using Fisher least significant difference procedure.

[**]Tospin version 2.1, Bruker BioSpin srl, Milan, Italy.

†† Amix software, version 3.8.4, Bruker BioSpin, Milan, Italy.

Multivariate statistical analysis tools were applied to study the metabolomics profiles of GCP, GAgP and HI groups.[28] The supervised statistical procedure applied for data reduction and classification was a combination of Principal Component Analysis (PCA) and canonical correlation analysis (CA) on the PCA scores. K-nearest neighbors (kNN) learning method (k = 5) applied on the CA scores was used to predict test samples. The global accuracy for classification was assessed by means of a Monte Carlo cross-validation scheme. Twenty-two metabolites, corresponding to well defined and resolved peaks in the spectra, were assigned. Signal identification was performed using a library of NMR spectra of pure organic compounds, public databases (e.g. HMBD) storing reference. The relative concentrations of the various metabolites in the different spectra were calculated by spectral fitting and integration of the signal area using in-house scripts[‡‡].[29] The Wilcoxon test was used for the determination of the statistically relevant metabolites. False discovery rate correction (FDR) was applied using the Benjamini and Hochberg method[30]: an adjusted *P*-value ≤ 0.05 was considered statistically significant. The changes in metabolites levels between periodontitis and healthy controls spectra were calculated as the $\log_2$ fold change (FC) ratio of the normalized median intensities of the corresponding signals in the spectra of the two groups. A statistical software program was used for pathway analysis[§§].[31]

Results

Characteristics of the study population

Demographic and clinical characteristics of the 100 participants according to the periodontal diagnosis are shown in supplementary Table 1 in the online *Journal of Periodontology*. The mean age in the GAgP group was significantly less than the other two groups, whereas ages were similar for the

[‡‡] Matlab and Statistics Toolbox, Mathworks Inc., Natick, Massachusetts, USA

[§§] MetaboAnalyst version 3.0, www.metaboanalyst.ca

GCP and HI groups ($P > 0.05$). Smokers in all the three clinical groups smoked less than 10 cigarettes a day (range 5 to 8).

As expected, the mean Full-Mouth Plaque Scores (FMPS), Full-Mouth Bleeding Scores (FMBS), PD, and CAL values were statistically significantly higher in patients with GAgP and GCP compared with the HI; all reached $P < 0.001$. When GCP and GAgP groups were compared, the only statistically significant difference found was in FMPS ($P < 0.001$).

Metabolomic profiling of saliva

The clustering of HI, GCP and GAgP patients was achieved by using PCA/CA on the ^{1}H-NMR profiles of saliva samples (Fig. 1). These analyses revealed 81% predictive accuracy discriminating HI from GCP, and 81% discriminating HI from GAgP affected patients (Fig. 2A,B). Permutation test (number of permutations = 1000) results showed statistically significant classification accuracy ($P < 0.001$). The statistical model applied proved to be effective to discriminate HI from GCP and GAgP patients, while the same statistical approach was not effective to discriminate GCP from the GAgP counterpart (60% predictive accuracy). Indeed, GAgP and GCP saliva samples seem belonging to the same metabolic space (Fig. 3). The predictive accuracy of these models did not change when smokers were excluded from the analysis (81% HI vs. GCP, 81% HI vs. GAgP, 60% GAgP vs. GCP).

Metabolites contributing to periodontal disease

The discrimination obtained between saliva samples of GCP and GAgP patients compared with HI, also demonstrated the existence of an altered metabolism in patients affected from periodontitis. Assigned signals in NMR spectra were integrated to obtain the concentration of metabolites in arbitrary units. By comparing the spectra of the saliva samples of periodontitis patients with HI, it results that GCP patients are characterized by lower levels ($P < 0.05$) of pyruvate, *N*-acetyl groups and lactate, and higher levels ($P < 0.05$) of proline, phenylalanine, isoleucine, valine and tyrosine, as summarized in Fig. 4A and Table 1. Compared with HI, GAgP

patients are characterized by lower levels ($P < 0.05$) of pyruvate, *N*-acetyl groups and lactate and sarcosine, and higher levels ($P < 0.05$) of formate, phenylalanine and tyrosine (Fig. 4B). A simplified list of the most contributing metabolic pathways is reported in Table 2. The analysis showed alteration in biochemical pathways like phenylalanine metabolism (phenylalanine, pyruvic acid, tyrosine, lactic acid) and pyruvate metabolism (pyruvic acid and lactic acid). The analysis was calculated based on adjusted *P*-value ($P < 0.05$) of the pathway enrichment analysis and an "Impact" (calculated from pathway topology analysis) equal to or greater than 0.1 was considered significant.

Discussion

The present study was designed to test the ability of NMR-based metabolomics to differentiate the biochemical signatures of GCP and GAgP in human saliva. To this purpose, gingivitis and localized manifestations of periodontitis were pointedly excluded because of the risk of flawing the results, and only periodontally HI were selected as controls. However, while corroborating substantial differences between pathological and healthy periodontal conditions, the multivariate analysis of NMR spectra did not provide a significant discrimination between the GCP and GAgP metabolomics profiles. The latter finding is in agreement with an increasing body of evidence and confirms that it is almost impossible to use the term AgP as long as there is no proper way to diagnose the disease.[32] Indeed, the discrimination between CP and AgP is not supported by sufficiently distinct histological, microbiological, immunological or genetic foundations.[33-37] Moreover, microbiome exhibits conserved metabolic and virulence gene expression profiles despite the inter-individual differences in the disease phenotype.[38] This may suggest that what distinguishes AgP from CP are dissimilarities in the immune-inflammatory host response[39] or, as advocated by Van der Velden[32], a difference in the degree of bacterial invasiveness. It is unlikely that the sole analysis of the metabolites in oral fluids can detect any pathognomonic benchmarks. Presumably, as far as all new high-throughput technologies have proven the existence of several molecular signatures in distinct periodontal patients[35], the traditional binomial classification seems not to fit this emerging heterogeneity anymore.

New models need to be hypothesized and tested, but due to the disease complexity a simultaneous multiomics approach should be elected.

The secondary goal was assessing the potential of oral fluid-based metabolomics to provide robust molecular biomarkers for periodontal diagnosis. Chronic periodontal infections activate the patient's host response to liberate a myriad of metabolic products at the interface between the tooth and the periodontal pocket.[13] The discrimination between the salivary samples from periodontitis patients, irrespective of the type of disease, and HI strengthens the evidence of a metabolomics trace of periodontitis in human saliva.[17] The Human Metabolome Database reports about 800 metabolites detected in saliva. However, according to similar studies, the NMR spectral profiles of this set of subjects are dominated by the signals of 20−30 molecules.[16-18] The values of the relative concentrations of saliva metabolites were estimated through the integration of the signals in the NMR spectra and were found consistent with the results and the biological interpretation of a previous publication of this same group.[18] The significantly reduced levels of lactate detected in the saliva of patients with GCP are partially explained by its conversion to acetate and propionate by some of the most prevalent periodontal bacterial species.[40,41] This may reflect on the pyruvate concentrations as the result of the substrate depletion of the L-lactate dehydrogenase. The levels of proline, phenylalanine, isoleucine, valine and tyrosine were higher in the saliva samples of patients with GCP with respect to C, as the amounts (not significant) of fatty acids, dipeptides and monosaccharide. This parallel up-regulation of the lipase, protease and glycosidase activities found in periodontitis is responsible for the overall tissue degradation and offers an ideal environment for bacterial proliferation and immune cells migration.[42]

The results of the present study should be interpreted with caution, as there are some limitations. One of the major problems with oral metabolites is that there is no way to determine their true origin. They could be essential constituent of the patient unique saliva, they could derive from the breakdown of the host tissues as from the bacterial communities, even from the supragingival plaque. Kuboniwa et

al.[22] performed supragingival scaling prior to sample collection and found that the discriminating ability of their model was significantly improved. By the way, in this study a scaling session was not performed; being able to find differences in a largely noisy environment could have more impact on the development of a rapid, noninvasive diagnostic tool.

Furthermore, the groups were matched for gender and smoking habits, yet not for age. This was not possible due to the difference in the age of onset of the two clinical forms of periodontitis, therefore GAgP patients were in average younger than GCP patients and the control group was comparable for age only with GCP individuals. Although there are no NMR-based metabolomics studies on the effect of aging on saliva, it is well-known that aging has a drastic effect on the serum metabolome.[43,44] Concentrations of certain small molecule metabolites in saliva, including some hormones and many pharmaceuticals and drugs of abuse, are known to correlate quantifiably with concentrations in serum. Nonetheless, data from a companion study demonstrated that the salivary NMR fingerprint had low discrimination accuracy between young (14 - 40 years) and elderly (58 – 73 years).[18]

Finally, due to the limited number of light smokers within this subset, the effect of smoking habits on metabolomics profiling was not specifically analyzed. However, when smokers were excluded from the analysis, the discrimination accuracy of the predicting models remained unchanged. It was thus plausible to exclude smoking as a confounding factor. This finding corroborates previous data demonstrating that light smoking had a negligible effect on the salivary profile.[18]

A major challenge in clinical periodontology is to find a reliable molecular marker of tissue destruction with high sensitivity, specificity and utility. At present, there is still a certain level of noise in metabolites fluctuation occurring in the periodontal microenvironment during the pathogenesis of periodontitis, that is not currently understood.[45] The hurdle in identifying neat pathological phenotypes is due to the intrinsic heterogeneity of periodontal diseases and the inherent complexity underlying.[35,46] Presumably, the main concern a clinician should have is not about discriminating

between GCP and GAgP, but about the prompt detection of active or inactive phases of supporting tissue breakdown. Future endeavors of salivary biomarkers inquiries should be hence directed towards the real time assessment of disease activity and the molecular characterization of different phenotypes of severe periodontitis. Regarding this issue, metabolomics could be of most interest in future research directions.

Conclusions

This cross-sectional investigation provided the evidence that NMR-based metabolomics failed to detect an unequivocal biochemical signature discriminating GCP from GAgP. The absence of evidence is not automatically evidence of the absence, but this finding adds consistency to the quest to redefine the current classification of periodontitis. Conversely, the successful differentiation between healthy and diseased individuals corroborated the sensibility of metabolomics profiling as a source of potential panels of biomarkers for molecular diagnostics. Nonetheless, the complex multifactorial etiology of periodontitis will require large clinical trials bringing together a multiomics assessment of saliva to properly address this issue.

Acknowledgments

The authors received no financial support and declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Figure legends

Figure 1. PCA/CA score plot built on saliva spectra of healthy (HI) and periodontitis patients (GAgP and GCP). Healthy individuals are very well recognized, while the saliva samples of chronic and aggressive periodontitis are confounded within the same metabolic space as suggested by cross-validation result.

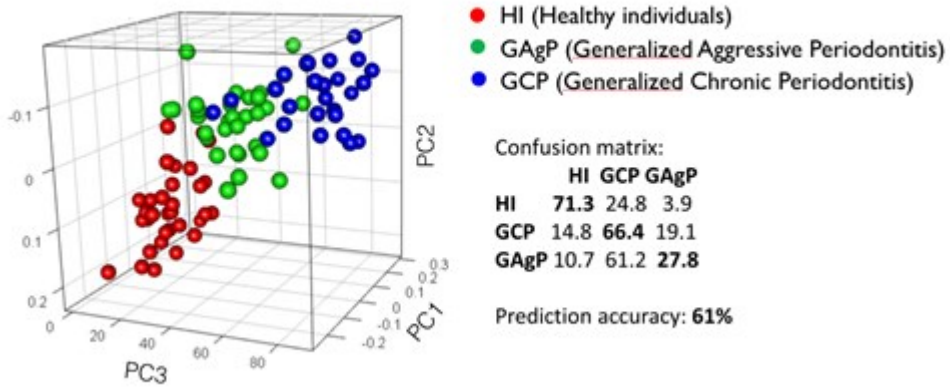

Figure 2. PCA/CA score plots. Discrimination between saliva spectra of healthy individuals and chronic periodontitis patients (A) and between healthy and aggressive periodontitis patients (B). In both cases, the discrimination is effective as it arises from the related prediction accuracy.

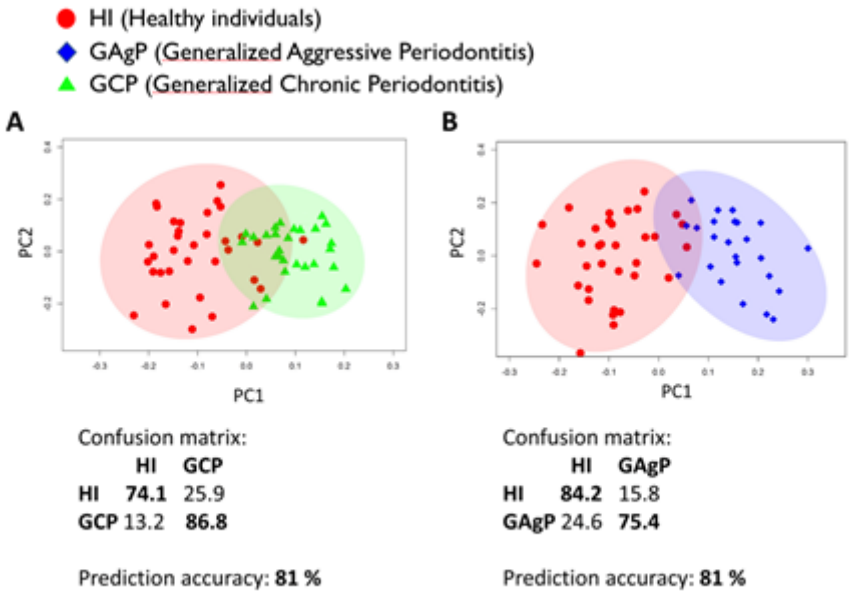

Figure 3. PCA/CA score plot. Discrimination between saliva spectra of chronic and aggressive periodontitis subjects. The model is not effective in discriminating the two groups (60% predictive accuracy).

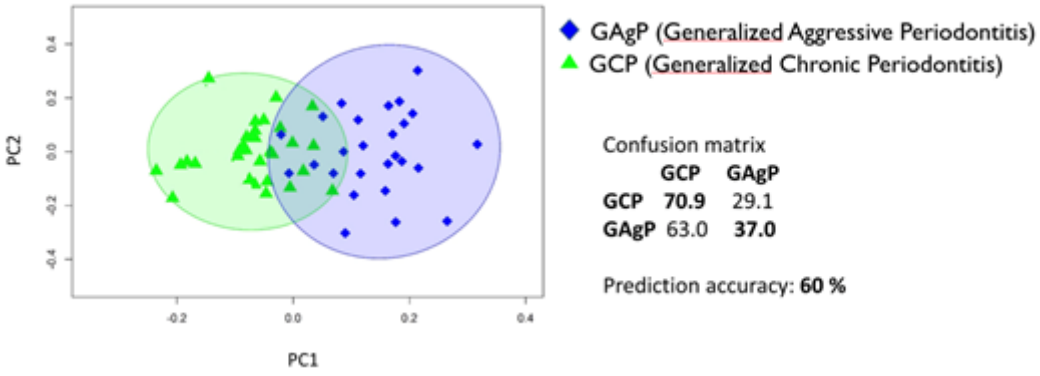

Figure 4. Changes in metabolites levels between healthy individuals and chronic periodontitis (A) and between healthy and aggressive periodontitis (B) calculated as the $\log_2$ Fold Change (FC) ratio of the normalized median intensities of the corresponding signals in the spectra of the two groups. Green bars represent significantly altered metabolites ($P < 0.05$).

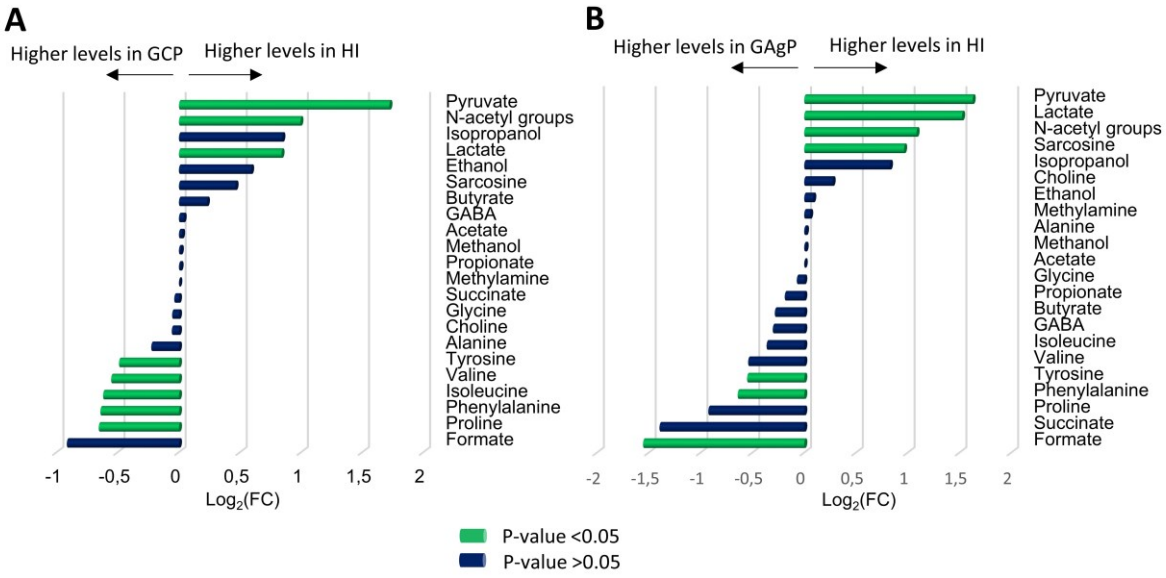

Table 1. Metabolites that result discriminant [median ± mean absolute deviation (MAD), in arbitrary units] between healthy individuals (HI) and patients with GCP and GAgP.

Variable	HI Group (*n*=39)	GCP Group (*n*=33)	FDR* HI vs. GCP	GAgP Group (*n*=28)	FDR* HI vs .GAgP
Isoleucine	0.131 ± 0.064	0.202 ± 0.075	**0.0029**	0.169 ± 0.071	0.4120
Valine	0.079 ± 0.04	0.115 ± 0.075	**0.0037**	0.114± 0.081	0.166
Propionate	0.598 ± 0.207	0.592 ± 0.265	0.9720	0.681 ± 0.544	0.3395
Isopropanol	0.05 ± 0.048	0.028 ± 0.03	0.1308	0.028 ± 0.041	0.2916
Ethanol	0.055 ± 0.045	0.037 ± 0.032	0.1999	0.052 ± 0.053	0.8935
Lactate	0.249 ± 0.62	0.139 ± 0.059	**0.0007**	0.087 ± 0.108	**0.0044**
Alanine	0.21 ± 0.083	0.245 ± 0.107	0.1920	0.207 ± 0.152	0.9922
Butyrate	0.048 ± 0.032	0.041 ± 0.031	0.9031	0.058 ± 0.043	0.5957
Acetate	13.503 ± 3.046	13.267 ± 4.108	0.9412	13.462 ± 5.744	0.8209
N-acetyl-groups	0.747± 0.463	0.375 ± 0.229	**0.0086**	0.352 ± 0.451	**0.0481**
Proline	0.045 ± 0.033	0.071 ± 0.046	**0.0221**	0.085 ± 0.059	0.1171
Pyruvate	0.272± 0.343	0.082± 0.05	**0.0001**	0.088 ± 0.063	**0.0044**
Succinate	0.065 ± 0.126	0.067 ± 0.082	0.9720	0.171 ± 0.124	0.33956
Methylamine	0.023 ± 0.011	0.023 ± 0.011	0.9031	0.022 ± 0.011	0.8714
Sarcosine	0.026 ± 0.02	0.019± 0.012	0.2134	0.013 ± 0.007	**0.0086**
GABA	0.128 ± 0.066	0.125 ± 0.085	0.9637	0.158 ± 0.113	0.6568
Choline	0.197 ± 0.103	0.205± 0.069	0.9412	0.163 ± 0.146	0.8483
Methanol	0.083 ± 0.179	0.082 ± 0.07	0.9720	0.083± 0.177	0.8209
Glycine	0.626 ± 0.205	0.65 ± 0.303	0.1920	0.657 ± 0.549	0.6707
Tyrosine	0.057 ± 0.0210	0.08 ± 0.02	**0.0297**	0.083 ± 0.037	**0.0481**
Phenylalanine	0.092 ± 0.0412	0.143 ± 0.044	**0.0007**	0.1430 ± 0.044	**0.0099**
Formate	0.0045 ± 0.021	0.009 ± 0.0684	0.1197	0.0134 ± 0.073	**0.0086**

*False discovery rate correction. Bold face indicates statistically significant inter-group differences.

Table 2. An integrated analysis based on MetaboAnalyst software: view of most contributing pathways.

Pathway Name	P-value	Holm P-value	FDR*	Impact
Phenylalanine metabolism	5.95×10^{-5}	0.005	0.005	0.12
Pyruvate metabolism	0.002	0.127	0.03	0.32

*False discovery rate correction.