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Article

Changes in the salivary metabolic profile of generalized periodontitis patients after non-surgical periodontal therapy. A metabolomic analysis using nuclear magnetic resonance spectroscopy

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Abstract: Pattern analysis of salivary metabolic profile has been proven accurate to discriminate generalized periodontitis (GP) patients from healthy individuals (HI) as disease modifies the salivary concentrations of specific metabolites. Due to the scarcity of data in the literature, the aim of this study was to determine whether non-surgical periodontal therapy (NST) could change salivary metabolomic profile in GP to one more similar to HI. Unstimulated whole saliva of 11 HI and 12 GP patients were obtained prior to and 3 months after NST. Metabolic profiling was performed using Nuclear Magnetic Resonance (NMR) spectroscopy, followed by supervised multivariate statistical approach on entire saliva spectra and partial least square (PLS) discriminant analysis. In GP group, periodontal treatment improved all clinical parameters, but not all the diseased sites were eradicated. PLS revealed an accuracy of 100% in discriminating the metabolomic profile of each GP patient before and after NST. OPLS was able to discriminate the 3 groups of subjects with an accuracy of 85.6%. However the post-NST metabolic profile of GP patients could not be completely assimilated to that of HS. Although NST may produce significant changes in the metabolic profile, GP patients maintained a distinctive fingerprint compared to HI.

Keywords: diagnosis; metabolomics; periodontal therapy; periodontitis; saliva.

1. Introduction

Periodontitis is a chronic inflammatory disease that leads to loss of periodontal attachment, resorption of alveolar bone, and, if left untreated, to tooth loss. It is spread worldwide and affects a large proportion of adult subjects [1]. Periodontitis is caused by a dysbiosis between the host and the bacterial biofilm that colonizes the dental surfaces. Conventional non-surgical periodontal therapy (NST) consists of oral hygiene instruction, patient motivation and mechanical removal of supra- and sub-gingival periodontal biofilm deposits [2]. The mechanical removal of the biofilm is effective in improving clinical parameters, reducing inflammation and restoring the host-microbial symbiotic state [3,4]. At present, the diagnosis of periodontitis (together with the assessment of treatment outcome) is primarily based on clinical and radiographic examination [5].

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Gingival crevicular fluid (GCF) and saliva have been investigated in the attempt to provide a detailed understanding of the molecular changes associated with periodontal tissue destruction [6]. Saliva has already proved to be a viable biofluid for the identification of alterations occurring in systemic and oral health status [7]. It is composed by a high variety of locally synthetized and systemically derived molecules involved in various metabolic processes and it is influenced by the interactions that occur in between the patient, the microbiome, and environmental factors [8,9]. Importantly the salivary metabolome is easily accessible and has revealed specific metabolomics signatures for numerous oral or systemic diseases [10]). NMR-based metabolomics provided significant information on oral conditions such as dental caries [11], oral cancer [12], and Sjögren's syndrome [13] as well as on systemic conditions such as dementia [14], neurodegenerative disorders [15] and cardiovascular diseases [16].

As a consequence metabolomics has recently emerged as a relevant approach for the investigation of the phases (i.e. onset, progression, treatment response) of periodontal diseases [17]. It deals with the high-throughput identification and quantification of metabolites in biological fluids by means of Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS). Several studies have demonstrated that the metabolic signature of saliva samples collected from chronic periodontitis patients is different from that of healthy controls, reaching discrimination accuracies up to 84% [1,18,19]. Metabolites defining the periodontal status are associated with either bacterial metabolic activity (lactate, pyruvate, formate) [18] or tissue degradation (proline, phenylalanine, tyrosine) [20,21], or even host immune response (valine, isoleucine) [22].

It was also proposed that salivary biomarkers could be useful for monitoring the response to NST, in terms of changes in salivary content of pro-inflammatory cytokines and proteinases [23-26].

This study aimed to determine whether the salivary metabolic profile of patients with generalized severe periodontitis changes after NST and if the metabolomic profile of treated patients is similar to that of periodontally healthy patients.

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2. Experimental Section

2.1. Study design and population

Participants were consecutively recruited at C.I.R. Dental School, University of Turin (Italy) during the period from April to September 2018. The protocol of the study was approved by the Institutional Ethical Review Board (Protocol n° 1503/2016) and the study was conducted in accordance with the Helsinki Declaration (as revised in 2002). Written informed consent was obtained from all participants.

Patients were included in the study if the presented with generalized stage III periodontitis (GP) with at least 20 natural teeth, ≥ 30% of teeth with clinical attachment level (CAL) of ≥ 5 mm and presence of bleeding on probing (BoP). No more than 4 teeth should have been lost due to periodontitis and the complexity factors defining the need for a complex rehabilitation should not be satisfied [5,27]. Furthermore healthy individuals (HI) that had at least 20 natural teeth, no interdental CAL loss at > 1 non-adjacent tooth, PD ≤ 3 mm, ≤ 10% of sites with BoP, and no radiographic evidence of bone loss [29] were selected among the undergraduate students of the C.I.R. Dental School.

Subjects in both groups were non-smokers. None had received periodontal treatment over the previous 6 months or antibiotic treatment within the previous 3 months.

Individuals suffering from systemic conditions including cardiovascular diseases, diabetes, and other systemic disorders that could affect the periodontal tissues or could impact the metabolic profile were excluded. Subjects using medications with a confirmed side effect on periodontal tissues such as phenytoin, cyclosporine, anti-inflammatory drugs, or calcium channel blockers were also excluded. None of the women were pregnant or in lactation period.

2.2 Clinical Examination and Periodontal Treatment

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At baseline, all participants underwent a periodontal examination by one experienced periodontist who was previously calibrated (95.3% intra-examiner concordance). Clinical measurements were taken at six sites per tooth for all teeth (excluding third molars), using a North Carolina periodontal probe (Hu-Friedy, IL, USA). At the same sites, plaque levels [29] and BoP were recorded dichotomously as present or absent. The number and percentage of PDs ≥ 4 mm and PDs ≥ 6 mm, the number of teeth, FMPS and FMBS were recorded.

After clinical and radiographic examination, GP patients underwent a session of supragingival scaling and received oral hygiene instructions. Subgingival scaling and root planing (SRP) were performed by a single trained periodontist on a quadrant-wise protocol using hand instruments (Gracey curettes, Hu-Friedy) together with ultrasonic devices (Cavitron Select, Dentsply, York, PA, USA). Mechanical therapy was completed within 28 days. After the end of mechanical treatment, patients were recalled every month for supragingival prophylaxis and oral hygiene reinforcement. The periodontal parameters described above were recorded again 3 months after the completion of mechanical therapy.

2.3 Saliva sampling collection

To prevent blood contamination, saliva samples were collected between 9:00 and 11:00 a.m. on the day following the clinical examination. Participants must avoid eating, drinking, using mouthwash, and brushing their teeth for 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 polypropylene tube for 10 min. A minimum of 1.0 ml of unstimulated saliva was collected into a sterile graduated polypropylene tube and immediately frozen at -80°C. In GP group, saliva sampling was repeated 3 months after NST.

2.4 Salivary metabolomics profiling

Frozen saliva samples were thawed at room temperature and were centrifuged (5000 g for 30 minutes at 4°C) to remove debris and to precipitate cells. 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 added to 300 µl of the supernatant, and the mixture was homogenized by vortexing for 30 seconds. An aliquot of 450 µl of this mixture was transferred into a 4.25 mm NMR tube (Bruker Biospin srl, Milan, Italy) for analysis. Monodimensional ^1H NMR spectra were acquired using a standard pulse sequence (noesygpprd.comp, Bruker Biospin) using 128 scans, receiver gain 11.3, 65,536 data points, a spectral width of 12,019 Hz, relaxation time of 4 s, and a mixing time of 0.1 s. working at 300 K using a Bruker 600 MHz spectrometer. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated using a RMN processing software (Tospin 2.1, Bruker BioSpin srl, Milan, Italy). Spectra were aligned by calibrating the TMSP peak at 0.00 ppm and segmented into 0.02 ppm chemical shift bins (water resonance region from 4.3 ppm to 6.5 ppm was excluded from the bins), and the corresponding spectral areas were integrated using AMIX software (Bruker BioSpin, version 3.8.4) [30,31]. Total area was calculated on the bins and total area normalization was carried out on the data prior to pattern recognition.

2.5 Statistical analysis

All data analyses were performed using R statistical package. The significance of changes in clinical data with time in GP group was determined using the paired t-test. An unpaired t-test was applied to test the differences between periodontitis and healthy subjects.

Multivariate partial least square (MPLS) analysis was applied to compare the metabolic profile of the HI group with that of GP patients before and after NST [32], using a combination of Principal Component Analysis (PCA) and canonical correlation analysis (CA). The accuracy for classification was assessed employing a Monte Carlo cross-validation scheme. All metabolites were assigned

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according to the available literature and reference databases (e.g. HMDB, BIOREFCODE) [10,33]. The relative concentration of the various metabolites in the different spectra was calculated by spectral fitting and integration of the signal area [34]. Mann–Whitney U test and Wilcoxon signed-rank test were chosen to infer metabolite differences between HI and GP patients before treatment and between GP patients before and after treatment, respectively, based on the assumption that metabolites are not normally distributed in the salivary samples. Multiple comparisons were addressed by post-hoc test. The changes in metabolites levels between periodontitis and healthy control spectra were calculated as the log2 fold-change ratio of the normalized median intensities of the corresponding signals in the spectra of the two groups.

3. Results

Saliva samples were collected from 12 GP patients (7 females and 5 males and, mean age 62. ± 4.9 yrs.) and 11 HI (6 females and 5 males, mean age of 23.8 ± 0.4 yrs). Participants were balanced with respect to gender ($p > 0.05$).

Clinical parameters of HI and GP patients at baseline and after treatment are presented in Table 1. The number of remaining teeth was higher in HI ($p < 0.005$). As expected, the clinical conditions of GP patients were significantly worse than those of HI ($p < 0.001$). At re-evaluation none of the GP cases reported complications or adverse effects of treatment. FMPS and FMBS decreased significantly in GP group from baseline to 3 months ($p < 0.001$). At 3 months after therapy, no statistically significant differences were observed for FMPS values between the two groups. Periodontal treatment was also associated to a decrease in the mean percentage of sites with PD ≥ 4 mm and PD ≥ 6 mm ($p < 0.001$), even though they remained significantly higher than in the HI group ($p < 0.001$).

Table 1. Demographic and clinical parameters in healthy and periodontitis patients before and after non-surgical periodontal treatment

	Healthy Individuals (HI)		Generalized periodontitis (GP) patients			
			Baseline		After Treatment	
	Mean	SD	Mean	SD	Mean	SD
Age (years)	23.82	0.40	55.25	4.88	55.25	4.88
N. of teeth	27.73	2.33	26.67	1.44	26.67	1.44
N. of sites	166.36	13.97	160.00	8.61	160.00	8.61
N. of PD ≥ 4 mm	2.18	1.25	83.42	33.73	28.58	23.77
% of PD ≥ 4 mm	1.33	0.79	53.16	20.47	18.63	13.97
N. of PD ≥ 6 mm	0.00	0.00	19.25	14.86	5.67	3.92
% of PD ≥ 6 mm	0.00	0.00	12.46	10.44	3.68	2.66
FMPS (%)	7.45	3.39	86.84	14.34	27.39	10.48
FMBS (%)	7.36	2.66	76.79	18.51	28.17	9.39

FMPS, full-mouth plaque score; FMBS, full-mouth bleeding score; PD, probing depth; SD, standard deviation.

In order to evaluate the impact of the NST on the metabolic signature of GP, the supervised MPLS pairwise approach was applied. This statistical model seemed to be effective in discriminating the metabolic phenotype of each untreated GP patient from its treated counterpart with a predictive accuracy of 100% (Fig. 1).

Figure 1. Score plot of Multilevel PLS pairwise discrimination of baseline and post-treatment (after therapy) salivary samples in GP patients.

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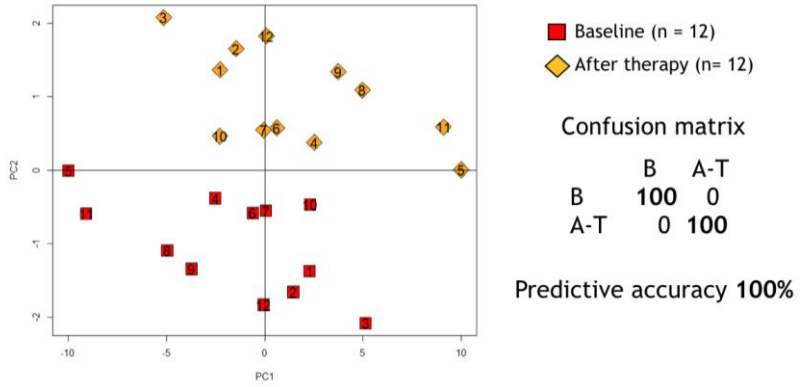
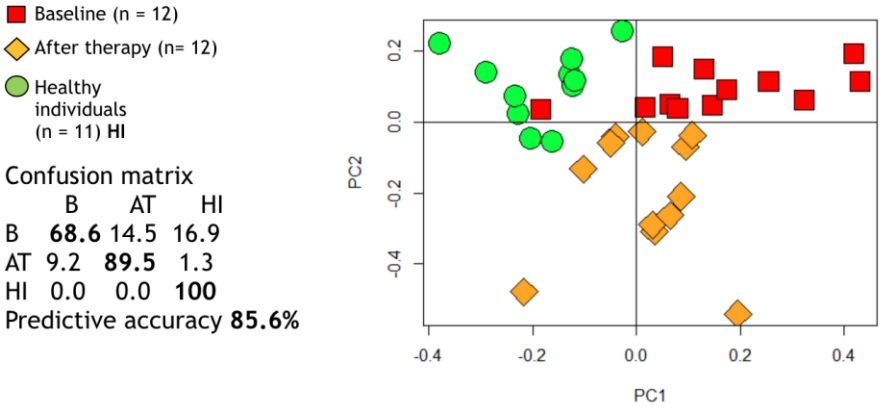


Furthermore, PCA/CA statistical approach was used to assess the separation of HI profiles from those of GP patients at baseline and 3 months after the completion of the non-surgical treatment, resulting in an overall discrimination accuracy of 85.6% (Fig. 2). As displayed GP treated patients occupied an intermediate metabolic space between their baseline counterpart and HI.

Figure 2. Projection of the ^{1}H NMR spectral buckets of the salivary samples into the two most significant dimensions of the PCA/CA considering the subspace for each healthy control (HI) and GP patient at baseline and after periodontal treatment.



Changes in individual metabolites among the three groups are reported in Table 2. When comparing HI and GP groups after treatment we observed reduced concentrations of acetate ($p = 0.004$) and lactate ($p = 0.012$) and increased concentrations of ethanol ($p = 0.036$), proline ($p = 0.016$), valine ($p = 0.009$), acetoin ($p = 0.002$), methylamine ($p = 0.006$), lactate ($p = 0.0011$), creatine ($p = 0.019$), and choline ($p = 0.023$) in the saliva of H subjects. Finally, comparing the saliva from S3 subjects before and after treatment we observed a reduction of the concentration of leucine ($p = 0.045$), valine ($p = 0.001$), phenylalanine ($p = 0.031$), isoleucine ($p = 0.014$), hypoxanthine ($p = 0.031$), and uracil ($p = 0.004$) and an increased concentration of succinate ($p = 0.033$) and formate ($p = 0.019$) at the completion of NST.

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Table 2. Salivary concentration of metabolites in healthy individuals and GP patients before and after periodontal treatment.

	Healthy Individuals (HI)		Generalized periodontitis (GP) patients			
			Baseline		After treatment	
	Mean	SD	Mean	SD	Mean	SD
acetate	731.26	324.31	9223.37	9223.37	119495.58	38614.20
propionate	9223.37	9223.37	9899.67	8369.22	18185.02	14457.12
glycine	16615.03	9427.33	5003.56	3304.07	4134.86	1717.69
butyrate	3252.59	1542.33	401.65	486.18	811.82	726.82
taurine	540.03	563.16	1486.88	910.20	1053.22	447.67
alanine	946.69	452.63	2310.43	2162.54	835.16	400.38
ethanol	529.93	325.07	148.08	112.61	294.00	248.31
proline	176.00	122.96	402.35	490.42	363.87	218.22
succinate	3052.11	3424.66	1169.95	2850.01	1783.53	1496.60
leucine	697.94	171.73	1615.23	1542.82	689.33	441.56
valine	551.16	245.92	1033.03	1033.37	305.23	335.29
isovalerate	438.91	235.40	437.92	451.65	536.68	453.33
isocaproate	315.28	274.43	131.81	152.28	222.04	144.81
acetoin	510.06	283.17	210.81	165.41	210.34	165.32
tyrosine	270.99	232.40	526.45	448.03	307.53	210.88
methylamine	274.34	96.61	289.01	355.64	169.15	73.67
phenylalanine	547.29	188.64	1183.61	1084.98	479.24	169.59
isoleucine	193.49	79.10	617.81	726.80	143.31	98.65
galactose	139.57	285.92	106.61	116.74	140.20	166.60
lactate	1349.14	1394.03	3954.64	2931.81	5413.35	5361.44
aspartate	58.25	37.36	166.05	100.56	106.12	94.69
creatine	494.94	558.86	306.27	300.30	226.62	413.65
choline	1372.24	526.06	805.57	827.51	879.44	908.50
methanol	637.30	739.04	596.06	327.05	1452.51	2117.43
pyruvate	1077.92	1722.88	614.20	613.88	961.51	731.33

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formate	513.89	799.40	795.86	1969.75	1656.13	2726.19
glucose	70.20	97.73	158.64	188.73	74.34	83.34
sarcosine	202.90	75.21	246.03	283.76	244.60	257.13
hypoxanthine	30.65	20.27	162.68	305.64	33.43	20.06
uracil	74.81	65.70	379.97	624.16	61.67	49.70
isopropanol	406.78	247.21	201.52	152.41	293.53	221.85

4. Discussion

The present study was designed to determine whether NST could change the salivary metabolomic profile of patients with generalized severe periodontitis.

As detected in the MPLS score plot NST induced a comprehensive shift in the salivary metabolome, probably due to the reduction of periodontal inflammation and to a significant change within the oral microbiome [35,36]. The accuracy of the multivariate model in discriminating the metabolomic profile of GP patients before and after treatment was 100%. Together with the metabolomic changes, the periodontal treatment led to a significant improvement in all recorded clinical parameters ($p < 0.001$). This improvement is in line with those reported in the literature [37]. During the study period patients were enrolled in a strict recall program for professional debridement and reinforcement of self-performed oral hygiene. This resulted in a low level (< 20%) of plaque scores through the study period.

Single metabolites fluctuation may be linked to different events occurring in health and disease and to the changes produced by treatment. The levels of valine, and isoleucine decreased after NST ($p < 0.005$) as they reflect host immune response to oral microbiome and have already been associated to active periodontal disease [22]. Parallel, uracil decreased as it has been related to BoP, PDs and CAL [38]. Importantly the reduction of the concentration of hypoxanthine after treatment confirms that NST is able to counterbalance the metabolic alteration occurring at cellular level as a consequence of bacterial insult. In fact, hypoxanthine concentration is related to bacteria-mediated purine degradation, a pathway a major biochemical source for reactive oxygen species production, that was significantly accelerated at the disease sites [39]. The increased concentration of formate is associated to a reduction in number of anaerobic bacteria present in the oral environment. The decreased phenylalanine could be a consequence of reduced host tissue degradation after NST [20,21].

Significant differences in specific metabolites were also found between healthy subjects and treated periodontitis patients. The lower concentration of lactate and the higher concentration of methylamine may be linked back to bacterial metabolism. The higher level of proline in treated periodontitis patients could be explained by the up-regulation of protease activity found in periodontal disease [39]. The higher level of choline in healthy patients is in agreement with the findings from García-Villaescusa et al. [40] who found a greater concentration of this metabolite in healthy patients compared to gingivitis/early periodontitis subjects. Finally, the lower level of acetate in treated individuals is in contrast with previous evidence [1,41] and yet difficult to interpret.

The second purpose of this investigation was to test the hypothesis that NST could restore the disease-related alterations in the individual salivary metabotype to a profile similar to that of periodontally healthy patients. Due to the obvious difficulties in collecting data before the onset of periodontal disease, we decided to compare the metabolome of post-treatment salivary samples with that of healthy subjects. Looking at the results of the combined PCA/CA analysis, the metabolic profile from treated periodontitis patients (3-month follow-up) occupied an intermediate region of the 3-dimensional plot, between that from healthy subjects and that from samples collected before non-surgical treatment. In agreement with our previous investigation [42], it is not possible to assimilate the metabolic profile of treated periodontitis patients to that of healthy subjects. One possible explanation could be that non-surgical periodontal treatment is not resolutive in all patients

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[43,44] and additional interventions, i.e. antimicrobials and/or surgical therapy, could be necessary to achieve all treatment goals [45,46]. Indeed, in the present investigation, most of the treated patients displayed some residual pockets and sites with persistent inflammation at the 3-month revaluation. The confounding effect of residual diseased sites could be overcome by comparing only pre- and post-treatment metabolic profiles of patients who achieve all treatment goals. Furthermore, 3 months could be a too short period to detect any relevant metabolic change after the completion of the mechanical treatment. Another explanation could be that periodontitis patients still hold a metabolic signature of their individual susceptibility to the disease even after successful non-surgical treatment. This speculation requires further investigation since it is still unclear whether individual susceptibility to periodontal disease lies in the local immune-inflammatory reactivity, in the microbiome, or at the systemic metabolic level [47,48]. In the future, it should be useful to compare pre- and post-treatment metabolomic profile of successfully treated patients (≤ 10% of sites with BoP, no periodontal pockets >4 mm with BoP or no deep periodontal pockets (≥6 mm) and the salivary metabolome of responders versus non-responders to periodontal treatment, possibly over a longer follow-up period.

5. Conclusions

Based on these data, NMR-spectroscopic analysis revealed that, despite significant changes in the overall metabolic fingerprint after NST, periodontitis patients maintained a distinctive metabolic profile compared to healthy individuals. Metabolic analysis could be used to monitor treatment stages and may discriminate patients with active or previous periodontitis from healthy individuals.

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