**Effect of non-surgical periodontal therapy on salivary metabolic fingerprint of generalized chronic periodontitis using nuclear magnetic resonance spectroscopy**



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Archives of Oral Biology 2019;97:208-214.
doi: *10.1016/j.archoralbio.2018.10.023*

**Effect of non-surgical periodontal therapy on salivary metabolic fingerprint of generalized chronic periodontitis using nuclear magnetic resonance spectroscopy.**

Federica Romano[a], Gaia Meoni[b], Valeria Manavella[a], Giacomo Baima[a], Giulia Maria Mariani[a], Stefano Cacciatore[c], Leonardo Tenori[d,1], Mario Aimetti[a,*,1]

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

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

[c]Cancer Genomics Research Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Cape Town, South Africa.

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

[1]The authors equally contributed to the work.

Corresponding author at*:* Department of Surgical Sciences, C.I.R. Dental School, Via Nizza 230 10126 Turin (Italy). Email address: mario.aimetti@unito.it

*Running title:* Metabolomic profile after non-surgical treatment

**ABSTRACT**

*Objective:* Metabolomic analysis of saliva proved its accuracy in discriminating patients with generalized chronic periodontitis (GCP) from healthy subjects by identifying specific molecular signatures of the disease. There is lack of investigations concerning the effect of periodontal treatment on individual metabolic fingerprints. Therefore, the aim of this study was to determine whether non-surgical periodontal therapy could change salivary metabolomic profile in GCP to one more similar to periodontal health.

*Design:* Unstimulated whole saliva of 32 controls and 19 GCP patients were obtained prior to and 3 months after conventional staged non-surgical periodontal therapy. Metabolic profiling was performed using Nuclear Magnetic Resonance (NMR) spectroscopy, followed by univariate and multivariate paired approaches to assess the changes introduced by the therapy.

*Results:* In GCP group, periodontal treatment led to an improvement in all clinical parameters ($p < 0.001$). The accuracy of the multivariate model in discriminating the metabolomic profile of each GCP patient at two time points was 92.5%. Despite the almost perfect separation of the spectra in the metabolic space, the univariate analysis failed to identify significant variations in single metabolite content. The post-treatment metabolic profile of GCP patients could not be assimilated to that of healthy controls who exhibited different levels of lactate, pyruvate, valine, proline, tyrosine, and formate.

*Conclusions:* Based on these data, NMR-spectroscopic analysis revealed that, despite significant changes in the overall metabolomic fingerprint after non-surgical therapy, GCP patients maintained a distinctive metabolic profile compared to healthy individuals.

*Keywords:* chronic periodontitis; metabolomics; periodontal/therapy; saliva.

## 1. Introduction

Saliva is an easily accessible biofluid and comprises a wide spectrum of both locally synthetized and systemically derived molecules involved in various biological functions (de Almenida Pdel, Grégio, Machado, de Lima, & Azevedo, 2008; Yoshizawa et al., 2013). Detecting changes in the concentration of these compounds allowed identification of individual metabolic fingerprints that can reflect many oral and systemic pathophysiological conditions (Dame et al., 2015; Silwood, Lynch, Claxson, & Grootveld, 2002). Among these conditions, periodontitis is a chronic immune-inflammatory disease with a complex pathophysiology, bringing together microbiological determinant and both host genetic and epigenetic variations (Jiao, Hasegawa, & Inohara, 2014; Kurgan & Kantarci, 2018). Irrespective of the differences among clinical phenotypes of periodontitis, the first phase of conventional periodontal therapy always involves bacterial plaque disruption and thorough cleaning of the root surfaces (Cobb, 2002). Although not resolutive in all treated cases, the mechanical removal of the periodontal biofilm has proven effective in controlling inflammation and, generally, in restoring the host-microbial symbiotic state (Mombelli, 2018). However, while advances in technology have introduced high-resolution techniques over the past decades, the determinants affecting the individual response to the treatment remain poorly understood and no biomarkers can accurately estimate the effectiveness of the periodontal therapy (Chambrone, Chambrone, Lima, & Chambrone, 2010; Gul et al., 2017).

Metabolomics is a newly emerging field of research dealing with the high-throughput identification and quantification of small-molecule metabolites in biological fluids and it has the potential to shed new lights into the multilevel complexity related to onset, progression and resolution of periodontitis (Zhang, Sun, Yan, Wang, & Wang, 2015). Nuclear magnetic resonance (NMR) spectroscopy analysis is considered as a robust, reproducible quantitative method (Duchemann et al., 2016) and proved its accuracy in discriminating patients with periodontitis from healthy subjects by identifying specific molecular signatures of the disease

in the salivary metabolic phenotype (Aimetti, Cacciatore, Graziano, & Tenori, 2012; Yoshizawa et al., 2013; Kuboniwa et al., 2016; Rzeznik et al., 2017).

This metabotype remains relatively stable over time and displays traits of individual specificity in periodontally healthy subjects, so there could be a potential way to distinguish which fluctuations from the individual fingerprinting are related to the disease (Wallner-Liebmann et al., 2016). NMR-based metabolomics has been successfully employed in different pathological context providing significant information on oral conditions such as dental caries (Fidalgo et al., 2013), periodontal disease (Aimetti, Cacciatore, Graziano, & Tenori, 2012; Romano et al., 2018), Sjögren's syndrome (Mikkonen et al., 2013) and oral cancer (Mikkonen et al., 2016) as well as on systemic conditions such as dementia (Figueira et al., 2016), neurodegenerative disorders (Yilmaz et al., 2017) and cardiovascular diseases (Ussher, Elmariah, Gerszten, & Dyck, 2016).

To date, no attempts have been performed to characterize the changes of human salivary metabotype after etiological periodontal treatment. Therefore, the present study was conducted to determine whether non-surgical periodontal therapy could change salivary metabolomic profile in patients affected by generalized chronic periodontitis (GCP) to one more similar to periodontal health. This would be important to lay the groundwork for understanding the molecular basis of individual response to periodontal treatment and for identifying molecular targets for diagnostic tests and novel therapies development.

## 2. Material and methods

### *2.1 Study design and population*

The GCP patients and periodontally healthy controls (H) participating in the study were recruited among the outpatients undergoing periodontal consultation or routine dental examination at the C.I.R. Dental School, Department of Surgical Sciences, University of Turin, Turin (Italy) between January and June 2017. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2002 and was approved by the local

Ethical Committee (Protocol n° 1503/2016). Written consent was obtained from all patients prior to the beginning of the study. Due to the lack of previous studies investigating changes in metabolic profile in GCF following periodontal treatment the power analysis was performed retrospectively. According to the Cohen formulation of power analysis (Cohen, 1988), and using Wilcoxon as the statistical test (with an alpha of 0.05, and a power of 80%), 32 controls and 19 GCP patients undergoing non-surgical therapy are enough to detect a medium-large effect-size *(Cohen's d* = 0.76). The study appears well powered for this explorative analysis.

The selection and diagnosis of participants was based on the clinical and radiographic criteria proposed by the 1999 International World Workshop for a Classification of Periodontal Disease and Conditions (Armitage, 1999). GCP patients were required to have at least 20 natural teeth and to demonstrate radiographic evidence of bone loss, clinical attachment level (CAL) of ≥ 5 mm in > 30% of intraoral sites and presence of bleeding on probing (BoP) (Lindhe et al., 1999). The severity of periodontal damage was in accordance with the local factors (Armitage, 1999).

The periodontally healthy group had at least 20 natural teeth, no probing depth (PD) and CAL > 3 mm, ≤ 15% of sites with BoP, and no horizontal or vertical bone loss in radiographic examination (Ertugrul, Sahin, Dikilitas, Alpaslan, & Bozoglan, 2013).

All individuals in both groups were in good general health, were non-smokers and none had received periodontal treatment over the previous 6 months or antibiotic medication within the past 3 months. No subjects with a history of systemic conditions such as heart disease, diabetes and other types of disorders that could affect the periodontal tissues or could impact the metabolic profile were included (Guash-Ferré et al., 2016). Enrolled individuals did not use medications that could affect the manifestations of periodontal disease, such as antibiotics, phenytoin, cyclosporine, anti-inflammatory drugs, or calcium channel blockers. None of the women were pregnant or in lactation period.

### *2.2 Clinical Examination and Periodontal Treatment*

All participants underwent a comprehensive periodontal examination by an experienced periodontist (GMM). After calibration, a 95.3% concordance within 1 mm for measurements of PD and CAL between the first and the second recording with an interval of 24 h was reached. Clinical measurements were taken at six sites per tooth for all teeth present (excluding third molars), using a North Carolina periodontal probe (Hu-Friedy, IL, USA). Plaque levels (plaque control record) (O'Leary, Drake, & Naylor, 1972), and BoP were recorded dichotomously as present or absent, and PD as well as CAL was measured in millimeter. Full-mouth periapical radiographs were taken with the long cone paralleling using Rinn holders.

After baseline examination, all GCP patients underwent a session of supragingival scaling and received instructions on oral hygiene techniques. One week later, they were subjected to quadrant-wise full-mouth subgingival scaling and root planing (SRP) under local anesthesia in four visits. Mechanical therapy was performed by a single trained periodontist (V.M.) using hand instruments (Gracey curettes, Hu-Friedy) and ultrasonic scalers (Cavitron Select, Dentsply, York, PA, USA) and was completed over a maximum of 28 days. Patients were followed every month for 3 months. Oral hygiene was reinforced and supragingival prophylaxis was carried out at each visit. Clinical monitoring was repeated 3 months post-therapy and the above detailed periodontal parameters were recorded again. All 3-month follow-up visits were completed in December 2017. All data were entered, and statistical analyses were performed at the completion of the 3-month study visits.

### *2.3 Saliva sampling collection*

The collection of saliva was done under standardized conditions, between 9:00 and 11:00 a.m., on the day following the clinical examination to prevent blood contamination and was repeated 3 months after non-surgical periodontal therapy for GCP patients (Holmes et al., 1994). Patients and controls were asked to refrain from eating, drinking, brushing and using

mouthwash for at least 1 h prior to saliva 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 and stored at -80°C until processing.

### *2.4 Salivary metabolomics profiling*

Frozen saliva samples were thawed at room temperature and centrifuged at 5000 *g* for 30 min to precipitate cells and remove any solid debris. 300 µl of the supernatant were added to 300 µl of sodium phosphate buffer (70 mm Na2HPO4; 20 % (v/v) $^{2}H_2O$; 6.15 mM NaN3; 6.64 mM sodium trimethylsilyl [2,2,3,3-$^{2}$H¬¬4] propionate (TMSP); pH 7.4). An aliquot of 450 µl of this mixture was transferred into a 4.25 mm NMR tube (Bruker Biospin srl, Milan, Italy) for analysis. For each saliva sample, a monodimensional $^{1}$H NMR spectrum was 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, a relaxation time of 4 s, and a mixing time of 0.1 s. working at 300 K using a Bruker 600 MHz spectrometer. Fourier transformed spectra were automatically corrected for phase and baseline distortions and calibrated using Topspin 2.1 (Bruker Biospin srl). Spectra were aligned to TMSP peak ($\partial_H$ 0.00 ppm). All spectra were 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) (Spraul et al., 1994; Holmes et al., 1994). 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 calculations were made using the R statistical environment. The Shapiro–Wilk test and Q–Q normality plots were applied to verify the normal distribution of the clinical quantitative variables. The significance of changes in clinical data with time in GCF group was

determined using the paired t-test. Differences between GCP and H groups were tested using the unpaired t-test.

Multivariate statistical analysis was applied to study the metabolomics profiles (Madsen, Lundstedt, & Trygg, 2010). The combination of Principal Component Analysis (PCA) and canonical correlation analysis (CA) on the PCA scores was used to discriminate H individuals from GCP patients before and after periodontal treatment. K-nearest neighbors (kNN) learning method (k = 5) was applied on the CA scores to predict test samples. The global accuracy for classification was assessed by means of a Monte Carlo cross-validation scheme.

All metabolites were assigned according to the available literature and reference databases (e.g. HMDB, BIOREFCODE) (Gardner, Parkes, Carpenter, & So, 2018; Wallner-Liebmann et al., 2016). The relative concentration of the various metabolites in the different spectra was calculated by spectral fitting and integration of the signal area (Wishart, 2008). Kruskal-Wallis test followed by Dunn post-hoc analysis test was chosen to infer metabolite differences among three groups (GCP before and after treatment, and H) on the biological assumption that metabolite concentrations are not normally distributed. False discovery rate correction (FDR) was applied using the Benjamini and Hochberg method (2000), an adjusted p-value $< 0.05$ was considered statistically significant.

The changes in metabolites levels between periodontitis and healthy control 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. In the GCP group a pairwise analysis was performed to follow the interindividual changes in metabolite levels after non-surgical periodontal treatment. Pairwise Wilcoxon test and multilevel partial least square (MPLS) were used with this aim (van Velzen et al., 2008; Westerhuis, van Velzen, Hoefsloot, Smilde, 2010).

**3. Results**

A flowchart of participants in the study is provided (Supplementary Figure 1). Saliva samples were collected from nineteen GCP patients (8 males and 11 females, mean age 48.9 ± 7.8 yrs.) and thirty-two controls (14 males and 18 females, mean age of 45.5 ± 7.0 yrs). Participants were balanced with respect to age and gender ($p > 0.05$).

Clinical parameters in GCP and healthy controls are presented in Table 1. The number of remaining teeth was similar in both groups. As expected, mean values of all periodontal parameters were significantly higher in GCP than in control group ($p < 0.001$).

The postoperative healing was uneventful in all GCP cases. As reported in Table 1, mean FMPS and FMBS decreased significantly in GCP 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 with a significant reduction in mean PD and mean CAL values ($p < 0.001$), but they remained significantly higher than in the H group ($p < 0.001$).

In order to evaluate the impact of the non-surgical periodontal therapy on the metabolic signature of GCP, a supervised MPLS pairwise approach was applied. This statistical model seemed to be effective in discriminating the metabolic phenotype of each untreated GCP patient from its treated counterpart with a predictive accuracy of 92.5% (Fig. 1). Furthermore, PCA/CA statistical approach was used to assess the separation of H controls profiles from those of GCP patients at baseline (Pre) and 3 months after the completion of the non-surgical treatment (Post), resulting in an overall discrimination accuracy of 82.2% (Fig. 2A) and 70% (Fig. 2B), respectively. As displayed in Figure 3, GCP treated patients occupied an intermediate metabolic space between their baseline counterpart and H individuals.

Changes in individual metabolites were generally modest when comparing salivary samples collected before and after therapy in GCP, while statistically significant differences in the concentrations of some molecules were identified in periodontitis patients after treatment compared to H controls. The main molecular markers for the observed differences were

valine, lactate, proline, pyruvate, tyrosine, and formate (Table 2, Supplementary Table 1 and Supplementary Figure 2).

**4. Discussion**

To the best of our knowledge, this was the first study conducted in any population regarding the impact of non-surgical periodontal treatment on the salivary metabolic fingerprint of GCP. Our focus on metabolites as biomarkers is based on the fact that metabolites are the final downstream products of the genome and the closest reporters of the functional phenotype of the organism (Dettmer, Aronov, & Hammock, 2007; Kuehnbaum & Britz-McKibbin, 2013). Metabolomics looks more workable than the analysis of the proteome or transcriptome because it deals with < 10,000 estimated small molecules in contrast to the millions or tens of thousands of proteins, transcripts and genes. Furthermore, changes in metabolites can be significant even when alterations in the concentrations of proteins and transcripts could not be detectable. Finally, metabolites permit to monitor the pathophysiology of both the host (the individual) and the guest (the microbiome), and their interactions. For this reason, metabolites are susceptible to environmental and external perturbations, even if their levels show a background signature that has proven to be individual and disease-specific (Wallner-Liebmann et al., 2016).

A defined metabolic signature of GCP has been detected in a number of investigations based on the metabolic profile of saliva samples of GCP patients compared to healthy controls, reaching discrimination accuracies up to 84% (Aimetti, Cacciatore, Graziano, & Tenori, 2012; Kuboniwa et al., 2016; Rzeznik et al., 2017). In agreement with previous findings from our group (Aimetti et al., 2012; Romano et al., 2018), metabolites defining the periodontal status mainly reflect bacterial species (lactate, pyruvate, formate) (Rzeznik et al., 2017) and tissue degradation (proline, phenylalanine, tyrosine) (Barnes et al., 2010; Holme, Huss, & Jeong, 2003), moreover they can describe host immune response (valine, isoleucine) (Zhang, Zeng, Ren, Mao, & Qiao, 2017).

Selected salivary biomarkers could have utility for monitoring periodontal health and response to therapy (Lee, Chen, Tu, Wu, & Chang, 2018). Previous investigations demonstrated changes in salivary content in terms of pro-inflammatory cytokines and proteinases as a result of non-surgical periodontal treatment (Kaushik, Yeltiwar, & Pushpanshu, 2011; Sanchez, Miozza, Delgado, & Busch, 2013; Sexton et al., 2011). In the present investigation the conventional quadrant-wise non-surgical therapy induced a comprehensive shift in the salivary metabolome of GCP patients as detected in the MPLS score plot. With MPLS approach, the between-subject variations were removed and only the within-subject treatment-related changes were taken into account (van Velzen et al., 2008; Westerhuis et al., 2010). The discrimination accuracy was high, of the order of 92.5%. This result is biologically plausible and can be likely ascribable to a significant quantitative and qualitative change within the oral microbiome and to the overall reduction of periodontal inflammation (Califf et al., 2017; Klukowska et al., 2015). The achieved improvements in clinical inflammatory and disease parameters were in line with those reported in the literature (Hung & Douglass, 2002). Plaque scores were maintained at a low level (<15%) through the study period due to the strict recall program for reinforcement of oral hygiene measures and professional debridement combined with self-perfomed plaque control.

Interestingly, intra-individual samples collected before and 3 months after treatment were accurately discriminated in the multivariate analysis, but no individual metabolite resulted *per se* statistically significant in the univariate analysis. While the explanation could lie in the sample size, consistent with the explorative nature of the study, it should be taken into account the extreme heterogeneity and variability within the complex interplay of molecules sustaining the pathogenesis of periodontitis. Given the impossibility to reduce this complexity to any single biomarker (Lee, Ghaname, & Braun, 2012) untargeted metabolomics appears to be a promising candidate to study the within- and between-subject differences in the salivary samples through a multiparametric approach (Barnes et al., 2014). In this case the individual

fingerprint could represent the best choice to describe the global response resulting from the sum of microbiome infection and individual immune response (Ebersole et al., 2016).

This investigation has also been undertaken to test the hypothesis that periodontitis leads to an imbalance in the individual salivary metabotype that could be partially restored by treatment. Although this could be best corroborated by collecting data before the onset of periodontal disease, due to the inherent difficulties, we compared the salivary metabolome of post-treatment samples with that of a subset of healthy subjects having similar characteristics except for periodontal status.

From the results of the unbiased PCA/CA analysis, it can be inferred that healthy and post-treatment periodontitis samples were projected into distinct metabolomic regions within the 3-dimensional plot. Unlike our expectations, the group of treated patients occupied an intermediate area between the healthy controls and their untreated counterpart. Looking at these data, it did not seem possible to assimilate the treated GCP patients to healthy subjects. Two possible mechanisms can be hypothesized to account for this evidence. First, SRP is not usually resolutive against periodontitis and the success of the non-surgical periodontal treatment remains highly unpredictable (Armitage & Xenoudi, 2016; Chambrone, et al., 2010). Most of the patients in this sample displayed some sites with persistent inflammation and residual pockets at 3-month revaluation. Indeed, it could also be speculated that 3 months may represent a too short time to detect any relevant metabolic difference. Furthermore, other strategies, i.e. adjunctive delivery of antimicrobials and surgical therapy, may be needed to reach the ultimate goals of periodontal treatment (Becker et al., 2001; Lindhe & Nyman, 1975). The comparison between pre and post-treatment metabolomic profiles focusing only on patients who completely satisfied with the treatment outcomes would presumably allow for ruling out the confounding effect of residual disease after non-surgical therapy.

The second speculation could be that, even though successfully treated, periodontitis patients still hold in their salivary metabotypes a signature of their background susceptibility to the

disease. Although a tempting hypothesis, experimental evidences are needed to make it more consistent. Whether this susceptibility lies in the microbiome, in the local immune-inflammatory reactivity or at the systemic metabolic level it is still unknown (Ebersole et al., 2016; Giannobile, 2012).

These findings warrant further investigation. We believe that future research should compare the salivary metabolomes of responders *versus* non-responders to periodontal treatment with the aim of both elucidating the factors responsible for the differential healing phenotype and estimating the specific risk profile of every patient.

**5. Conclusion**

NMR-spectroscopic analysis revealed significant changes in the overall metabolomic signature of periodontitis in saliva after the initial non-surgical therapy. However, this effect was not enough to shift the disease-related biochemical profile towards the salivary fingerprint associated with healthy periodontal status. The relatively small sample size, together with the high level of variability in human system biology, likely contributed to the low number of observed differences achieving statistical significance. It remains also not clear which is the exact contribution that microbiome, inflammatory mediators and host specific metabolic signatures (i.e. susceptibility) play in determining the intra and interpersonal variability in the salivary metabotype.

Understanding the molecular variations between periodontal health and disease in saliva could lead to the development of more sensitive diagnostic tools and more appropriate interventional strategies for periodontitis. Due to the complex multifactorial etiology of periodontitis, clinical trials with larger sample size are needed to add consistency and external validity to the present results.

**Funding**

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Conflicts of interest**

The authors declare that they have no conflicts of interest.

**Ethical approval**

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the ethical committee of the "AOU Città della Salute e della Scienza" (Turin, Italy) (Ref: 1503/2016).

**References**

Aimetti, M., Cacciatore, S., Graziano, A., Tenori, L. (2012). Metabonomic analysis of saliva reveals generalized chronic periodontitis signature. *Metabolomics, 8*, 465–474.

Armitage, G. C. (1999). Development of a classification system for periodontal diseases and conditions. *Annals of Periodontology, 4*, 1–6.

Armitage, G. C., Xenoudi, P. (2016). Post-treatment supportive care for the natural dentition and dental implants. *Periodontology 2000*, *71*, 164–184.

Barnes, V. M., Kennedy, A. D., Panagakos, F., Devizio, V., Trivedi, H. M., Jonsson, T., et al. (2014). Global metabolomic analysis of human saliva and plasma from healthy and diabetic subjects, with and without periodontal disease. *PLoS One*, *9*, e105181.

Barnes, V. M., Teles, R., Trivedi, H. M., Devizio, W., Xu, T., Lee, D. P., et al. (2010). Assessment of the effects of dentifrice on periodontal disease biomarkers in gingival crevicular fluid. *Journal of Periodontology*, *81*, 1273–1279.

Becker W, Becker BE, Caffesse R, Kerry, G., Ochsenbein, C., Morrison, E., et al. (2001). A longitudinal study comparing scaling, osseous surgery, and modified Widman procedures: results after 5 years. *Journal of Periodontology, 72*, 1675–1684.

Benjamini, Y., Hochberg, Y. (2000). On the adaptive control of the false discovery rate in multiple testing with independent statistics. *Journal of Educational and Behavioral Statistics, 25*, 60–83.

Califf, K. J., Schwarzberg-Lipson, K., Garg, N., Gibbons, S. M., Caporaso, J. C., Slots, J., et al. (2017). Multi-omics analysis of periodontal pocket microbial communities pre- and post-treatment. *mSystems, 2*, e00016–17.

Chambrone, L., Chambrone, D., Lima, L. A., Chambrone, L. A. (2010). Predictors of tooth loss during long-term periodontal maintenance: a systematic review of observational studies. *Journal of Clinical Periodontology*, *37*, 675–684.

Cobb, C. M. (2002). Clinical significance of non-surgical periodontal therapy: an evidence-based perspective of scaling and root planing. *Journal of Clinical Periodontology*, *29(suppl 2)*, 6–16.

Cohen, J. (1988). *Statistical power analysis for the behavioral sciences.* (2nd ed.). Hillsdale, NJ: Erlbaum.

Dame, Z. T., Aziat, F., Mandal, R., Krishnamurthy, R., Bouatra, S., Borzouie, S., et al. (2015). The human saliva metabolome. *Metabolomics, 11*, 1864–1883.

de Almenida Pdel, V., Grégio, A. M., Machado, M. A., de Lima, A. A., Azevedo, L. R. (2008). Saliva composition and functions: a comprehensive review. *The Journal of Contemporary Dental Practice, 9*, 72–80.

Dettmer, K., Aronov, P. A., Hammock, B. D. (2007). Mass spectrometry-based metabolomics. *Mass Spectrometry Reviews*, *26*, 51–78.

Duchemann, B., Triba, M. N., Guez, D., Rzeznik, M., Savarin, P., Numes, H., et al. (2016). Nuclear magnetic resonance spectroscopic analysis of salivary metabolome in sarcoidosis. *Sarcoidosis Vasculitis and Diffuse Lung Diseases*, *33*, 10–16.

Ebersole, J. L., Graves, C. L., Gonzales, O. A., Dawson, D. 3rd, Monford, L. A., Huja, P. E., et al. (2016). Aging, inflammation, immunity and periodontal disease. *Periodontology 2000*, *72*, 54–75.

Ertugrul, A. S., Sahin, H., Dikilitas, A., Alpaslan, N., Bozoglan, A. (2013). Comparison of CCL28, interleukin-8, interleukin-1ß and tumor necrosis factor-alpha in subjects with gingivitis, chronic periodontitis and generalized aggressive periodontitis. *Journal of Periodontal Research*, *48*, 44–51.

Fidalgo, T. K. S., Freitas-Fernandes, L. B., Angeli R., Muniz A. M. S., Gonzlaves, E., Santos, R., et al. (2013). Salivary metabolite signatures of children with and without dental caries lesions. *Metabolomics*, *9*, 657–666.

Figueira, J., Jonsson, P., Nordin Adolfsson, A., Adolfsson, R., Nyberg, L., Öhman, A. (2016). NMR analysis of the human saliva metabolome distinguishes dementia patients from matched controls. *Molecular bioSystems, 12,* 2562–2571.

Gardner, A., Parkes, H. G., Carpenter, G. H., So, P. W. (2018). Developing and standardizing a protocol for quantitative proton nuclear magnetic resonance ($^1$H RMN) spectroscopy of saliva. *Journal of Proteome Research, 17*, 1521-1531.

Giannobile, W. V. (2012). Salivary diagnostics for periodontal diseases. *Journal of American Dental Association, 143(suppl 10)*, 6–11.

Guasch-Ferré, M., Hruby, A., Toledo, E., Clish, C. B., Martinez-Gonzalez, M. A., Salas-Salvadò, J., et al. (2016). Metabolomics in prediabetes and diabetes: a systematic review and meta-analysis. *Diabetes Care, 39*, 833–846.

Gul, S. S., Griffiths, G. S., Stafford, G. P., Al-Zubidi, M. I., Rawlinson, A., Douglas, C. W. I. (2017). Investigation of a novel predictive biomarker profile for the outcome of periodontal treatment. *Journal of Periodontology*, *88*, 1135–1144.

Holme, P., Huss, M., & Jeong, H. (2003). Subnetwork hierarchies of biochemical pathways. *Bioinformatics, 19,* 532–538.

Holmes, E., Foxall, P. J. D., Nicholson, J. K., Neild, G. H., Brown, S. M., Beddell, C. R., et al. (1994). Automatic data reduction and pattern recognition methods for analysis of $^{1}H$ nuclear magnetic resonance spectra of human urine from normal and pathological states. *Analytical Biochemistry, 220*, 284–296.

Hung, H. C., Douglass, C. W. (2002). Meta-analysis of the effect of scaling and root planing, surgical treatment and antibiotic therapies on periodontal probing depth and attachment loss. *Journal of Clinical Periodontology, 29*, 975–986.

Jiao, Y., Hasegawa, M., Inohara, N. (2014). The role of oral pathobionts in dysbiosis during periodontitis development. *Journal of Dental Research, 93*, 539–546.

Kaushik, R., Yeltiwar, R. K., Pushpanshu, K. (2011). Salivary interleukin-1beta levels in patients with chronic periodontitis before and after periodontal phase I therapy and healthy controls: a case-control study. *Journal of Periodontology*, *82*, 1353–1359.

Klukowska M, Goyal CR, Khambe D, Cannon, M., Miner, M., Gurich, N., et al. (2015). Response of chronic gingivitis to hygiene therapy and experimental gingivitis. Clinical, microbiological and metabonomic changes. *American Journal of Dentistry,* 28, 273–284.

Kuboniwa, M., Sakanaka, A., Hashino, E., Bamba, T., Fukusaki, E., Amano, A. (2016). Prediction of periodontal inflammation via metabolic profiling of saliva. *Journal of Dental Research, 95*, 1381–1386.

Kuehnbaum, N. L., & Britz-McKibbin, P. (2013). New advances in separation science for metabolomics: resolving chemical diversity in a post-genomic era. *Chemical Reviews*, *113*, 2473–2468.

Kurgan, S., Kantarci, A. (2018). Molecular basis for immunohistochemical and inflammatory changes during progression of gingivitis to periodontitis. *Periodontology 2000*, *76*, 51–67.

Lee, A., Ghaname, C. B., Braun, T. M., et al. (2012). Bacterial and salivary biomarkers predict the gingival inflammatory profile. *Journal of Periodontology, 83*, 79–89.

Lee, C.-H., Chen, Y.-W., Tu, Y.-K., Wu, Y.-C., Chang, P.-C. (2018). The potential of salivary biomarkers for predicting the sensitivity and monitoring the response to nonsurgical periodontal therapy: A preliminary assessment. *Journal of Periodontal Research, 53,* 545–554.

Lindhe, J., Nyman, S. (1975). The effect of plaque control and surgical pocket elimination on the establishment and maintenance of periodontal health. A longitudinal study of periodontal therapy in cases of advanced disease. *Journal of Clinical Periodontology*, *2*, 67–79.

Lindhe, J., Ranney, R., Lamster, I., Charles, A., Chung, C., Flemmig, T., et al. (1999). Consensus report: chronic periodontitis. *Annals of Periodontology, 4*, 38.

Madsen, R., Lundstedt, T., Trygg, J. (2010). Chemometrics in metabolomics--a review in human disease diagnosis. *Analitica Chimica Acta, 659*, 23–33.

Mikkonen, J. J. W., Herrala, M., Soininen, P., Lappalainen, R., Tjäderhane, L., Seitsalo, H., et al. (2013). Metabolic profiling of saliva in patients with primary Sjögren's syndrome. *Metabolomics, 3*, 128.

Mikkonen, J. J. W., Singh, S. P., Herrala, M., Lappalainen, R., Myllymaa, S., Kullaa, A. M. (2016). Salivary metabolomics in the diagnosis of oral cancer and periodontal diseases. *Journal of Periodontal Research*, *51*, 431–437.

Mombelli, A. (2018). Microbial colonization of the periodontal pocket and its significance for periodontal therapy. *Periodontology 2000*, *76*, 85–96.

O'Leary, T.J., Drake, R.B., Naylor, J.E. (1972). The plaque control record. *Journal of Periodontology, 43*, 38.

Romano, F., Meoni, G., Manavella, V., Baima, G., Tenori, L., Cacciatori, L., et al. (2018) Analysis of salivary phenotypes of generalized aggressive and chronic periodontitis through nuclear magnetic resonance-based metabolomics. *Journal of Periodontology,* http://doi.org/10.1002/JPER.18-0097

Rzeznik, M., Triba, M. N., Levy, P., Jungo, S., Botosoa, E., Duchemann, B., et al. (2017). Identification of a discriminative metabolomic fingerprint of potential clinical relevance in saliva of patients with periodontitis using $^{1}$H nuclear magnetic resonance (NMR) spectroscopy. *PLoS ONE*, *12*, e0182767.

Sánchez, G. A., Miozza, V. A., Delgado, A., Busch, L. (2013). Salivary IL-1b and PGE2 as biomarkers of periodontal status, before and after periodontal treatment. *Journal of Clinical Periodontology, 40*, 1112–1117.

Sexton, W. M., Lin, Y., Kryscio, R. J., Dawson, D. R. 3rd, Ebersole, J. L., Miller, C. S. (2011). Salivary biomarkers of periodontal disease in response to treatment. *Journal of Clinical Periodontology, 38*, 434–441.

Silwood, C. J. L., Lynch, E., Claxson, A. W., Grootveld, M. C. (2002). 1H and (13)C NMR spectroscopic analysis of human saliva. *Journal of Dental Research***,** *81*, 422–427.

Spraul, M., Neidig, P., Klauck, U., Kessler, P., Holmes, E., Nicholson, J. K., et al. (1994). Automatic reduction of NMR spectroscopic data for statistical and pattern-recognition classification of samples. *Journal of Pharmaceutical and Biomedical Analysis, 12*, 1215–1225.

Ussher, J. R., Elmariah, S., Gerszten, R. E., Dyck, J. R. B. (2016). The emerging role of metabolomics in the diagnosis and prognosis of cardiovascular disease. *Journal of the American Colledge of Cardiology*, *27*, 2850–2870.

van Velzen, E. J. J., Westerhuis, J. A., van Duynhoven, J. P., van Dorsten, F. A., Hoefsloot, H. C. J., Jacobs, D. M., et al. (2008). Multilevel data analysis of a crossover designed human nutritional intervention study. *Journal of Proteome Research, 7*, 4483–4491.

Wallner-Liebmann, S., Tenori, L., Mazzoleni, A., Dieber-Rotheneder, M., Konrad, M., Hofmann, P., et al. (2016). Individual human metabolic phenotype analyzed by $^{1}$H NMR of saliva samples. *Journal of Proteome Research*, *15*, 1787–1793.

Westerhuis, J. A., van Velzen, E. J., Hoefsloot, H. C., Smilde, A. K. (2010). Multivariate paired data analysis: multilevel PLSDA versus OPLSDA. *Metabolomics, 6***,** 119–128.

Wishart, D. S. (2008). Quantitative metabolomics using NMR. *TrAC Trends in Analytical Chemistry, 27*, 228–237.

Yilmaz, A., Geddes, T., Han, B., Behado-Singh, R. O., Wilson, G. D., Imam, K., et al. (2017). Diagnostic biomarkers of Alzheimer's disease as identified in saliva using 1H NMR-based metabolomics. *Journal of Alzheimer's Disease, 58,* 355–359.

Yoshizawa, J. M., Schafer, C. A., Schafer, J. J., Farrell, J. J., Paster, B. J., Wong, D. T. (2013). Salivary biomarkers: toward future clinical and diagnostic utilities. *Clinical Microbiology Reviews***,** *26*, 781–791.

Zhang, A., Sun, H., Yan, G., Wang, P., Wang, X. (2015). Metabolomics for biomarker discovery: moving to the clinic. *BioMed Research International, 2015*, 354671.

Zhang, S., Zeng, X., Ren, M., Mao, X., Qiao, S. (2017). Novel metabolic and physiological functions of branched chain amino acids: a review. *Journal of Animal Science and Biotechnology, 8,* 10.

**Table 1 Clinical parameters (mean ± SD) in periodontally healthy individuals and patients with GCP before and after periodontal treatment.**

| | Healthy individuals (*n*=32) | GCP patients (*n*=19) | |
|---|---|---|---|
| **Parameters** | | **Baseline** | **3 months** |
| **Sites with BoP (%)** | 12.7 ± 2.2 | 58.4 ± 23.6[¶] | 13.7 ± 3.8[*] |
| **Sites with Plaque (%)** | 11.5 ± 3.2 | 50.1± 14.7[¶] | 17.6 ± 4.9[*¶] |
| **PD (mm)** | 2.3 ± 0.4 | 5.4 ± 0.8[¶] | 3.7 ± 0.4[*¶] |
| **CAL (mm)** | 2.4 ± 0.5 | 6.0 ± 0.9[¶] | 4.9 ± 0.7[*¶] |
| **N° sites with PD ≥ 5 mm** | 0 | 62.3 ± 15.3[¶] | 22.2 ± 8.1[*¶] |
| **N° teeth** | 27.0 ± 1.6 | 26.3 ± 2.3 | 25.9 ± 2.1 |

[*]P < 0.001 *versus* baseline.
[¶]P< 0.001 *versus* healthy controls.

GCP, generalized chronic periodontitis; BoP, bleeding on probing; PD, probing depth; CAL, clinical attachment level.

**Table 2 Metabolites that result discriminant (P-values and FDR adjusted P-values) between healthy individuals and GCP patients before and after periodontal treatment.**

| | **GCP patients at baseline vs after therapy** | | **GCP patients at baseline vs Healthy individuals** | | **GCP patients after therapy vs Healthy individuals** | |
|---|---|---|---|---|---|---|
| | paired Wilcoxon test | | Post hoc Dunn test | | | |
| | *P-value* | FDR* adj. *P-value* | *P-value* | FDR* adj. *P-value* | *P-value* | FDR* adj. *P-value* |
| Isoleucine | 0.495 | 0.95 | 0.0110 | **0.0330** | 0.0340 | **0.0510** |
| Valine | 0.832 | 0.95 | 0.0050 | **0.0120** | 0.0077 | **0.0120** |
| Propionate | 1.000 | 1.00 | 0.9100 | 0.9500 | 0.8600 | 0.9500 |
| Isopropanole | 1.000 | 1.00 | 0.0560 | 0.1700 | 0.1770 | 0.2600 |
| Ethanol | 0.304 | 0.95 | 0.2400 | 0.6600 | 0.4400 | 0.6600 |
| Lactate | 0.671 | 0.95 | 0.0027 | **0.0040** | 0.0006 | **0.0018** |
| Alanine | 0.393 | 0.95 | 0.0960 | 0.2400 | 0.9400 | 0.9400 |
| Butyrate | 0.587 | 0.95 | 0.1400 | 0.4200 | 0.7200 | 0.7200 |
| Acetate | 0.325 | 0.95 | 0.4700 | 0.6400 | 0.6400 | 0.6400 |
| N-acetyl-groups | 0.043 | 0.95 | 0.0947 | 0.1145 | 0.0005 | **0.0016** |
| Proline | 0.442 | 0.95 | 0.0307 | **0.0460** | 0.0074 | **0.0220** |
| Pyruvate | 0.702 | 0.95 | 0.0001 | **0.0003** | 0.0003 | **0.0005** |
| Succinate | 0.580 | 0.95 | 0.5200 | 0.5200 | 0.3900 | 0.5200 |
| Methylamine | 0.671 | 0.95 | 0.4000 | 0.6500 | 0.4300 | 0.6500 |
| Sarcosine | 0.468 | 0.95 | 0.0790 | 0.1180 | 0.0200 | 0.0590 |
| GABA | 0.776 | 0.95 | 0.5300 | 0.8000 | 0.3600 | 0.8000 |
| Choline | 0.832 | 0.95 | 0.4300 | 0.7100 | 0.7100 | 0.7100 |
| Methanol | 0.196 | 0.95 | 0.4200 | 0.8800 | 0.4400 | 0.6700 |
| Glycine | 0.865 | 0.95 | 0.4300 | 0.8800 | 0.3800 | 0.6400 |
| Tyrosine | 0.832 | 0.95 | 0.0101 | **0.0150** | 0.0098 | **0.0150** |
| Phenylalanine | 0.229 | 0.95 | 0.0009 | **0.0028** | 0.0470 | 0.0705 |
| Formate | 0.325 | 0.95 | 0.0732 | 0.1100 | 0.0052 | **0.0160** |

*False discovery rate correction. Bold face indicates statistically significant ($p < 0.05$) inter-group differences.

**Figure legends**

**Fig. 1** Score plot of Multilevel PLS (MPLS) pairwise discrimination of pre- (baseline) and post-treatment (after therapy) salivary samples in GCP patients. Both sensitivity and specificity are 92.5% in the confusion matrix.

**Fig. 2** Score plots of PCA/CA: A) model effective in discriminating healthy subjects (H) and GCP patients at baseline; B) model for the classification of H and post-treatment GCP patients. Confusion matrices and predictive accuracies are reported for both models.

**Fig. 3** Projection of the $^{1}$H NMR spectral buckets of the salivary samples into the three most significant dimensions of the PCA/CA considering the subspace for each healthy control (H) and GCP patient before and after periodontal treatment.

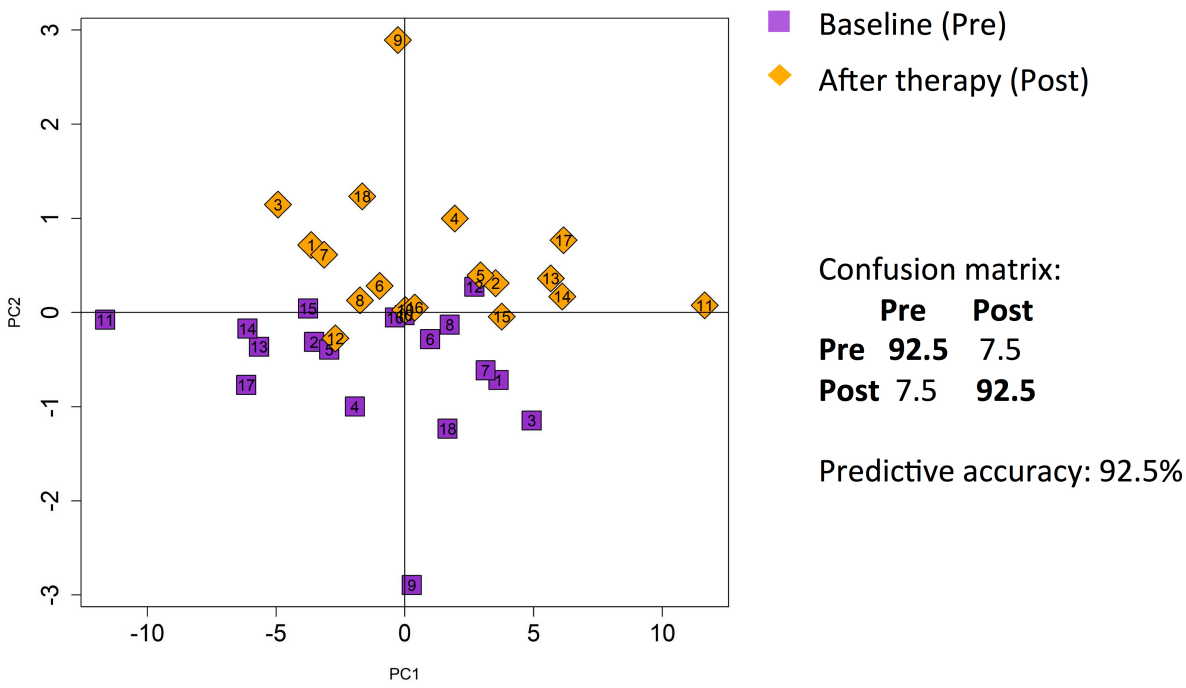
PC2
PC1
-10
-5
0
5
10
3
2
1
0
-1
-2
-3
Baseline (Pre)
After therapy (Post)
Confusion matrix:
Pre Post
Pre 92.5 7.5
Post 7.5 92.5
Predictive accuracy: 92.5%

**Figure 1.**

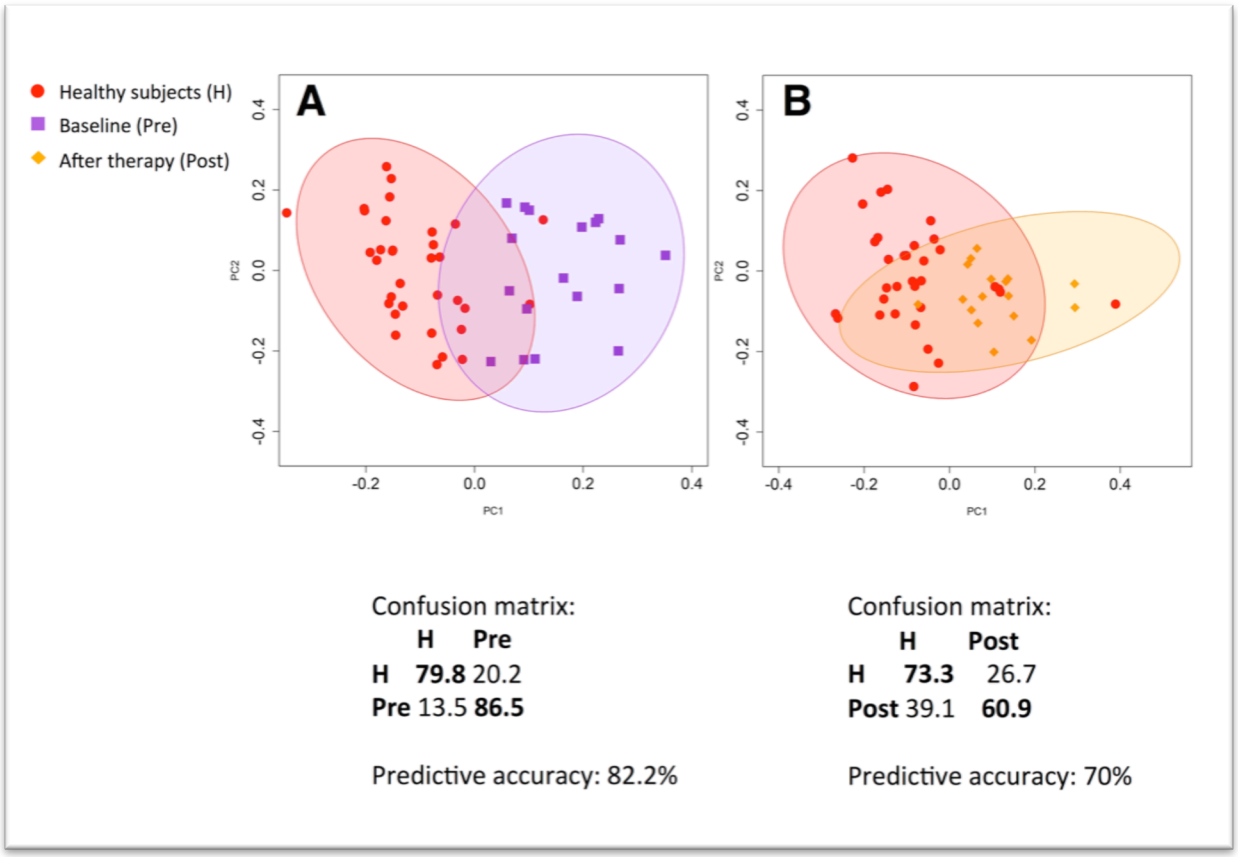
Healthy subjects (H)
Baseline (Pre)
After therapy (Post)
A
B
PC1
PC2
Confusion matrix:
H Pre
H 79.8 20.2
Pre 13.5 86.5
Predictive accuracy: 82.2%
Confusion matrix:
H Post
H 73.3 26.7
Post 39.1 60.9
Predictive accuracy: 70%

**Figure 2**

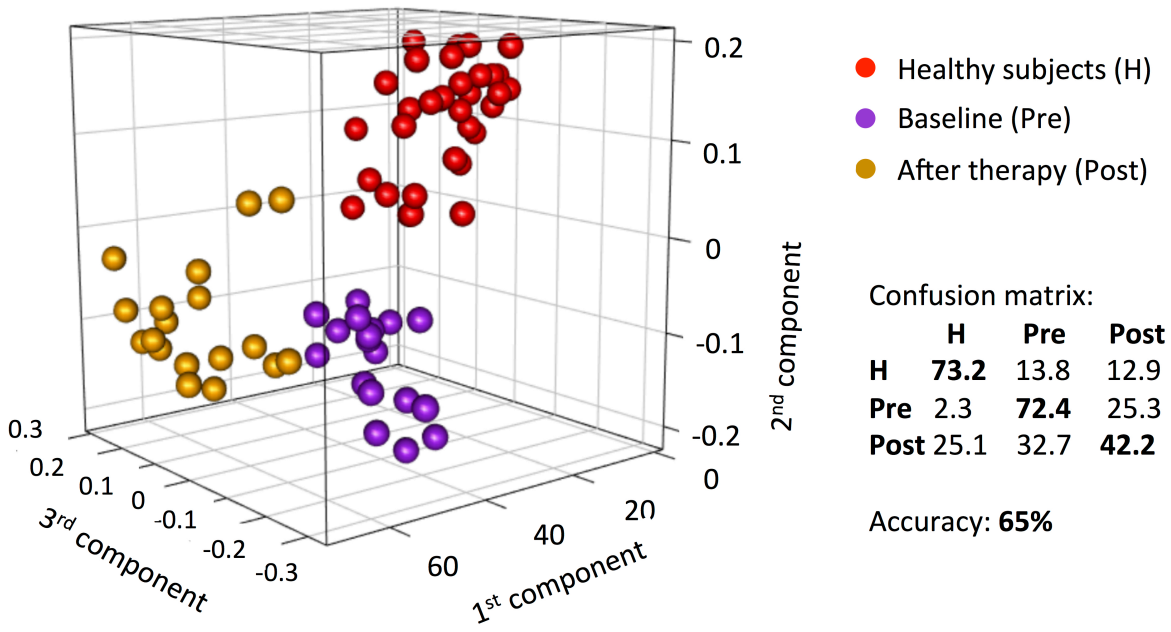
0.2
0.1
0
-0.1
-0.2
2nd component
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
3rd component
60
40
20
1st component
Healthy subjects (H)
Baseline (Pre)
After therapy (Post)
Confusion matrix:
H Pre Post
H 73.2 13.8 12.9
Pre 2.3 72.4 25.3
Post 25.1 32.7 42.2
Accuracy: 65%

**Figure 3.**