

RESEARCH ARTICLE

# Identification of a discriminative metabolomic fingerprint of potential clinical relevance in saliva of patients with periodontitis using <sup>1</sup>H nuclear magnetic resonance (NMR) spectroscopy

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## Abstract

Periodontitis is characterized by the loss of the supporting tissues of the teeth in an inflammatory-infectious context. The diagnosis relies on clinical and X-ray examination. Unfortunately, clinical signs of tissue destruction occur late in the disease progression. Therefore, it is mandatory to identify reliable biomarkers to facilitate a better and earlier management of this disease. To this end, saliva represents a promising fluid for identification of biomarkers as metabolomic fingerprints. The present study used high-resolution <sup>1</sup>H-nuclear magnetic resonance (NMR) spectroscopy coupled with multivariate statistical analysis to identify the metabolic signature of active periodontitis. The metabolome of stimulated saliva of 26 patients with generalized periodontitis (18 chronic and 8 aggressive) was compared to that of 25 healthy controls. Principal Components Analysis (PCA), performed with clinical variables, indicated that the patient population was homogeneous, demonstrating a strong correlation between the clinical and the radiological variables used to assess the loss of periodontal tissues and criteria of active disease. Orthogonal Projection to Latent Structure (OPLS) analysis showed that patients with periodontitis can be discriminated from controls on the basis of metabolite concentrations in saliva with satisfactory explained variance (R<sup>2</sup>X = 0.81 and R<sup>2</sup>Y = 0.61) and predictability (Q<sup>2</sup>Y = 0.49, CV-AUROC = 0.94). Interestingly, this discrimination was irrespective of the type of generalized periodontitis, *i.e.* chronic or aggressive. Among the main discriminating metabolites were short chain fatty acids as butyrate, observed in higher concentrations, and lactate, γ-amino-butyrate, methanol, and threonine observed in lower concentrations in periodontitis. The association of lactate, GABA,

and butyrate to generate an aggregated variable reached the best positive predictive value for diagnosis of periodontitis. In conclusion, this pilot study showed that  $^1\text{H}$ -NMR spectroscopy analysis of saliva could differentiate patients with periodontitis from controls. Therefore, this simple, robust, non-invasive method, may offer a significant help for early diagnosis and follow-up of periodontitis.

## Introduction

Periodontitis is characterized by the loss of the supporting tissues of the teeth in an inflammatory-infectious disease context [1–4]. The prevalence of periodontal disease remains high [5, 6]. Periodontitis is often diagnosed late, with important tissue damage already present and, if untreated, eventually leads to loss of teeth. Therefore, the main objective of periodontal treatment is to control infection in a sustainable manner and, secondarily, to repair damaged tissues. However, restoration of a “well-balanced host-microbial symbiotic state” [7–10] in the oral cavity at the level of periodontal structures may be difficult. Moreover, regeneration of diseased periodontal tissues by surgical or non-surgical techniques still remains hypothetical. In addition, while the replacement of lost tooth roots by dental implants can compensate for dental disability, high-risk patients for periodontal disease are also high-risk patients for implant therapy failure [11]. Finally, periodontitis may be associated with systemic diseases such as diabetes or cardiovascular disease [12, 13]. For all these reasons, the detection of early stage disease of periodontitis is essential. Currently, diagnosis relies on clinical and X-ray examination. Unfortunately, clinical signs of tissue destruction occur late, and clinical monitoring is time-consuming, subject to measurement error, and often poorly tolerated by patients. Therefore, it may be hypothesized that identifying precise and reliable biomarkers of disease activity would allow for better management of the disease.

Human saliva *per se* is a complex biological fluid secreted by major and minor salivary glands. In addition to true saliva components, oral fluid contains a wide spectrum of *compounds* originating from multiple local and systemic sources as a complex microbiota, serum and tissue components carried by the gingival crevicular fluid (GCF). [14–17]. GCF flows into the oral cavity from periodontal pockets and the intensity of this exudate, whose composition is close to that of serum, varies as a function of gingival inflammation [18]. In the present report, the term saliva is used to designate the collected oral fluid including all its components.

“Omics” methods are promising techniques in the field of system biology allowing a global evaluation of the metabolic changes in a biologic milieu. Indeed, if various reports dealt with “omics” in the saliva, they were mostly proteomic studies and less often metabolomic studies. Concerning periodontitis, it has been shown that “omics” technologies, such as proteomics, are particularly promising for periodontitis research [3, 19–25]. Recently, using a method based on gas chromatography and mass spectrometry on a set of 19 volunteers, Kuboniwa et al. showed that metabolic analysis of saliva in periodontitis may be of potential clinical interest [25]. Proton nuclear magnetic resonance ( $^1\text{H}$  NMR) spectroscopy analysis is considered as a robust, reproducible quantitative method having the ability to identify a maximal number, both hydrophilic and lipophilic metabolites via their unique spectral patterns referred as a metabolomics signature [26]. This method has been used to explore various physiological fluids in pathological conditions [27–29]. However, there have been only a few reports on  $^1\text{H}$  NMR spectroscopy investigations of saliva, almost exclusively for general diseases or physiological studies [30–35]. Indeed reports concerning periodontitis are extremely rare [25, 36,

37]. Therefore, we designed the present pilot study using  $^1\text{H}$  NMR spectroscopy of saliva with the goal of determining its potential clinical relevance through the identification of a metabolomics signature of active periodontitis in patients compared to healthy controls matched by age, gender, and smoking habits.

## Material and methods

### Ethics statement

The protocol was approved, under n°10–047, by the medical ethics international review board of Paris North Hospitals (N°IRB00006477) in accordance with the guidelines for the protection of human subjects. Written informed consent was obtained from all patients and controls prior to their participation.

### Study population and design

Twenty-six patients (10 men and 16 women) with chronic and aggressive generalized periodontitis (18 and 8 respectively) were recruited from among the outpatients at the Department of Periodontology of Bretonneau Hospital in Paris, France (AP-HP, University Paris Descartes, Paris, France). The diagnostic criteria for chronic and aggressive generalized periodontitis were defined according to the classification proposed at the International Workshop for the Classification of Periodontal Diseases and Conditions in 1999 [38]. Healthy controls ( $n = 26$ ) were simultaneously recruited from dental students or medical staff and were matched by age ( $\pm 5$  years), gender, and smoking habits. Both patient and control groups were balanced for ethnicity and socio-economic levels.

**Inclusion criteria.** The common inclusion criteria for patients with periodontitis or healthy controls were: (i) age range of 18–64 years (ii) health insurance benefit (iii) no systemic diseases, (iv) no antibiotics taken during the past three months, (v) no regular alcohol consumption and (vi) women not pregnant or nursing. Furthermore, for patients no initial therapy was applied within the 6 months before the study. After recruitment, patients and healthy controls who agreed to participate were asked to complete a questionnaire including the following sections: demographic and socio-economic data, medical history, dental habits and dental care utilization, and tobacco consumption. Smoking was quantified in pack-years, and patients' smoking habits were linearly stratified into 5 ordered categories [39, 40].

**Definition of periodontitis.** Periodontitis is defined as an active destruction of the periodontal tissues. The diagnosis was based on clinical and radiographic assessment: clinical measures of Pocket Depth (PD) and Clinical Attachment Loss (CAL), and evaluation of alveolar bone loss by X-ray [41, 42]. Moreover, a Bleeding On Probing (BOP) was included to characterize the degree of gingival inflammation, active periodontitis being not reflected only by attachment or bone loss measurements [43]. Periodontitis cases were defined according to CDC-AAP (Centers for Disease Control—American Academy of Periodontology) definitions updated in 2012 [42]. Observation of at least  $\geq 2$  interproximal sites with  $\text{CAL} \geq 3$  mm, and  $\geq 2$  interproximal sites with  $\text{PD} \geq 4$  mm (not on the same tooth) or one site with  $\text{PD} \geq 5$  mm was necessary to diagnosis. Periodontitis was diagnosed as localized if  $\leq 30\%$  and generalized if  $>30\%$  of the sites were affected [38].

According to the classification proposed at the International Workshop for the Classification of Periodontal Diseases and Conditions in 1999 [38], Chronic Periodontitis (CP) resulting from poor oral hygiene with the presence of bacterial plaque is prevalent in most adults. The rate of progression of attachment and bone loss is slow to moderate. In contrast, aggressive periodontitis (AP) is characterized by a rapid attachment loss and alveolar bone destruction in patients associated with varying degrees of bacterial plaque.

**Clinical data assessment.** The following parameters were appraised: Number of Residual Teeth (NRT) and Decay Missing Filled (DMF) for dental history; Plaque Control Record (PCR) [44] and Gingival Bleeding Index (GBI) [45] for presence of bacterial plaque and gingival inflammation. For patients with periodontitis, in a second clinical session after dental prophylaxis, indices assessing the severity and extent of the periodontitis i.e., PD, and CAL were recorded. Simultaneously, BOP was scored [46, 47]. Clinical parameters were assessed at four sites on each tooth (mesiobuccal, distobuccal, mesiolingual, and distolingual) using a manual periodontal probe (Hu-Friedy, Chicago, IL, USA), at 20 g of pressure. Tooth sites excluded from the examination were impacted teeth, retained roots, and teeth with indeterminable cemento-enamel junction. The third molar was excluded from analysis. For each site, CAL was distributed into groups (mild CAL: 3–4 mm, moderate CAL: 5–6 mm, and severe CAL:  $\geq$  6 mm) and their percentage was assessed for each patient. The highest value of tissue destruction was determined as CALMAX and the average CAL (CALMEAN) as well as the mean PD (MPD) were computed. The assessment of bone loss (BL) was performed on periapical radiographs of patients. Radiographs were taken using a standardized long-cone paralleling technique. Bone loss was measured according to the modified classification of Hugoson & Jordan [48]. For this study, a professional dentist trained for the evaluation and sampling procedure performed all clinical assessments and collections of saliva.

**Collection of stimulated saliva.** The collection of saliva was done under standardized conditions, between 09:00 and 11:00 am after paraffin wax-stimulation and to prevent blood contamination, before the assessment of clinical parameters. A volume of 10 mL saliva was collected ( $\pm$ 5 min), the pH was recorded and the sample immediately stored at  $-25^{\circ}\text{C}$ . Patients and controls were required to refrain from eating, drinking, chewing gum, and tooth brushing for at least 2h prior to the sample collection.

## $^1\text{H}$ NMR spectroscopy

For NMR analysis, samples were thawed at room temperature and centrifuged for 2 min at 2000g before analysis. A volume of 0.6 mL of saliva was placed into a 5-mm-diameter specific tube together with 0.1 mL of  $\text{D}_2\text{O}$ . The proton spectra were acquired at 500 MHz on a Varian Unity Inova<sup>®</sup> spectrometer at  $25^{\circ}\text{C}$ . A signal was acquired after a  $90^{\circ}$  pulse of 32K data points on a spectral window of 5000 Hz. The relaxation delay was 4s. The water signal was suppressed by a pre-saturation sequence using low-power irradiation (0.03 W for 2s) on the water-signal frequency during the relaxation delay. The resulting free induction decays obtained with 128 transients were processed by NMR Pipe software. A Fourier transformation was applied with an exponential window function to produce a 1 Hz broadening line. Spectra were phased and a multipoint linear baseline correction was applied. Each spectrum was referenced using a propionic acid signal (1.04 ppm). The spectral region between 0–9 ppm was divided into 9000 spectral regions of 0.001 ppm width, called buckets, using in-house C code. The water region was excluded [5.1; 4.3 ppm]. The icoshift tool for Matlab (Matlab<sup>®</sup> 2014, The Mathworks Inc., Natick, MA, USA) was used for realignment [49]. Each bucket was integrated and scaled using probabilistic quotient normalization [50].

## Statistical analysis

**Multivariate analysis.** Principal component analysis (PCA) was performed on clinical and NMR data. PCA was used to assess the homogeneity of the cohort and to detect and exclude any outliers defined as observations located outside the 95% confidence region of the model. For clinical data analysis, PCA was performed on the patient group to assess the homogeneity of the patient cohort regarding the clinical data and to detect any relationships between

clinical variables. This analysis was performed with the SIMCA-P statistical package (Umetrics, Umeå Sweden). For NMR data analysis, PCA was performed with an in-house Matlab<sup>®</sup> code using the same algorithm as SIMCA-P to detect any group separation based on signal variability.

An orthogonal projection to latent-structure (OPLS) analysis was run to discriminate patients and controls. Compared to the classical projection of latent-structure analysis (PLS), this method improves interpretation of the spectroscopic variations between discriminated groups by removing the orthogonal information that had no impact on the discrimination.

The goodness-of-fit parameters of the OPLS model R<sup>2</sup><sub>Y</sub> and Q<sup>2</sup><sub>Y</sub> were calculated. R<sup>2</sup><sub>Y</sub> represents the explained variation of the Y matrix. Q<sup>2</sup><sub>Y</sub> was calculated with the K-fold (K = 7) method and was used to estimate the predictability of the model. R<sup>2</sup><sub>Y</sub> = 1 indicates a perfect description of the data by the model while Q<sup>2</sup><sub>Y</sub> = 1 indicates a perfect prediction of new data. Score and loadings plots are used to illustrate the results. Each point in the score plot represents the projection of an NMR spectrum (and thus a control or patient sample) on the predictive (horizontal axis) and orthogonal components of the model (vertical axis). The loadings plot represents the covariance between the Y-response matrix and the signal intensity of the various spectral domains. Colours were also used in the loadings plot depending on correlations between the corresponding bucket intensity and the Y variable. Positive signals correspond to those metabolites that had an increased concentration in patients. Conversely, negative signals correspond to those metabolites that had an increased concentration in controls.

An internal validation of the OPLS model was performed using a permutation test (999 random permutations of group membership). The aim of this test was to evaluate whether our OPLS model, built with groups, were significantly better than any other OPLS model obtained by randomly permuting the original group attributions. The model is validated when R<sup>2</sup> and Q<sup>2</sup> are higher for the non-permuted compared to those of all permuted models.

To estimate the ability of the model to correctly classify a new data set, a receiver operating curve (ROC) was built using the predictions of each cross-validation set and an area under this curve was calculated (CV-AUROC).

**Multivariate logistic regression.** To study the risk associated with periodontitis (dependent variable), a descending stepwise multivariate logistic regression was performed. Prior to the logistic regression, a univariate analysis by a Mann–Whitney test was used to make the selection of the metabolites that will be computed for logistic regression analysis. Variables with  $P \leq 0.2$  on univariate analysis were included in the stepwise logistic regression analysis to compare patients to controls. For the multivariate phase,  $P < 0.05$  was considered statistically significant. [51].

## Results

A total of 52 subjects, 26 patients with generalized periodontitis (18 CP and 8 AP) and 26 controls were recruited. One control was not considered because of doubts concerning his periodontal health. A total of 25 controls were retained, one control being matched with 2 different cases.

Demographic characteristics of controls and patients and the clinical parameters of the patient group are summarized in Table 1. Clinical parameters were irrelevant for controls as they were healthy subjects free of any periodontitis.

The number of remaining teeth was similar between patients and controls ( $26 \pm 2.4$  vs  $26.6 \pm 2.13$ , NS). For patients, MPD and attachment loss (CAL) were  $3.82 \pm 0.5$  mm and  $4.12 \pm 0.78$  mm, respectively.

**Table 1. Clinical data of patients with periodontitis and controls.**

		periodontitis	controls
N <sup>1</sup>		26	25
	men	10	9
	women	16	16
Age	years (mean ± SD)	42.4 ± 12.8	40.7±12.4
Smoking habits <sup>1</sup>	none	13	12
	former	2	2
	current	11	11
NRT <sup>2</sup> (mean ± SD)		26 ± 2.4	26.6 ± 2.1
Generalized periodontitis <sup>3</sup>	chronic	18	0
	aggressive	8	
Severity index <sup>4</sup>	mild	1	/
	moderate	6	
	severe	19	
Mean DMF <sup>5</sup>		8.23	/
Mean affected sites (%)		48.5	/
Mean PCR <sup>6</sup> (%)		61.2	/
Mean BOP <sup>7</sup> (%)		35.0	/
PD <sup>8</sup> (mean ± SD, in mm)		3.8± 0.5	/
CAL <sup>9</sup> (mean ± SD, in mm)		4.1± 0.8	/
BL <sup>10</sup>	Grade 1	5	/
	Grade 2	12	
	Grade 3	9	

<sup>1</sup>smoking habits were stratified according to [39].

<sup>2</sup>NRT: number of residual teeth;

<sup>3</sup>controls were free of periodontitis;

<sup>4</sup>according to [42];

<sup>5</sup>DMF: decay missing filled;

<sup>6</sup>PCR:plaque control record;

<sup>7</sup>BOP:bleeding on probing;

<sup>8</sup>PD: pocket depth;

<sup>9</sup>CAL:clinical attachment loss;

<sup>10</sup>BL:bone loss [grade according to [48]

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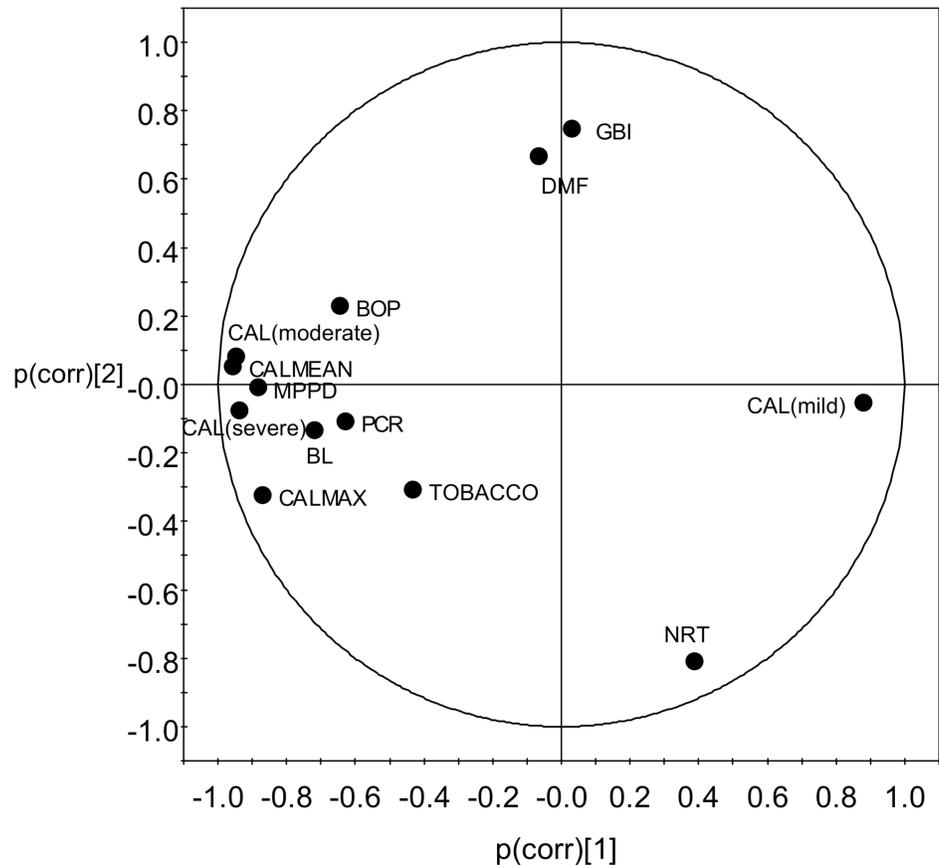
### Multivariate analysis of clinical data

PCA was performed on periodontal variables from the 26 patients. The score plot is shown in Fig 1.

No outlier was detected in the plan defined by the first and second principal components, PC1 and PC2, which correspond to 63% of the variability in the clinical data. Considering the positions of variables on the loadings plot, the loss of periodontal tissues (including CAL-MEAN, percentage of sites with severe or moderate CAL, BL), the criteria of active disease (MPD), and the percentage of bleeding sites (BOP) were strongly correlated and contribute strongly to PC1 variability.

### Multivariate analysis of metabolomics data

Two examples of NMR spectra obtained in saliva from a control individual and a patient case are respectively shown on Fig 2a and 2b.



**Fig 1. Loadings plot of the principal component analysis (PCA) performed on clinical variables of periodontitis.** Loadings are scaled so that the correlated variables correctly explained by the components are found close together and near the correlation circle. BL: bone loss; BOP: bleeding on probing; CAL: clinical attachment loss, expressed as a mean (CALMEAN) or according to the severity of the loss (CALmild, CALmoderate, and CALMAX); DMF: decay missing filled; MPPD: mean pocket depth; NRT: number of residual teeth; PCR: plaque control record; TOBACCO: smoking habits (for details see [Materials and methods](#)).

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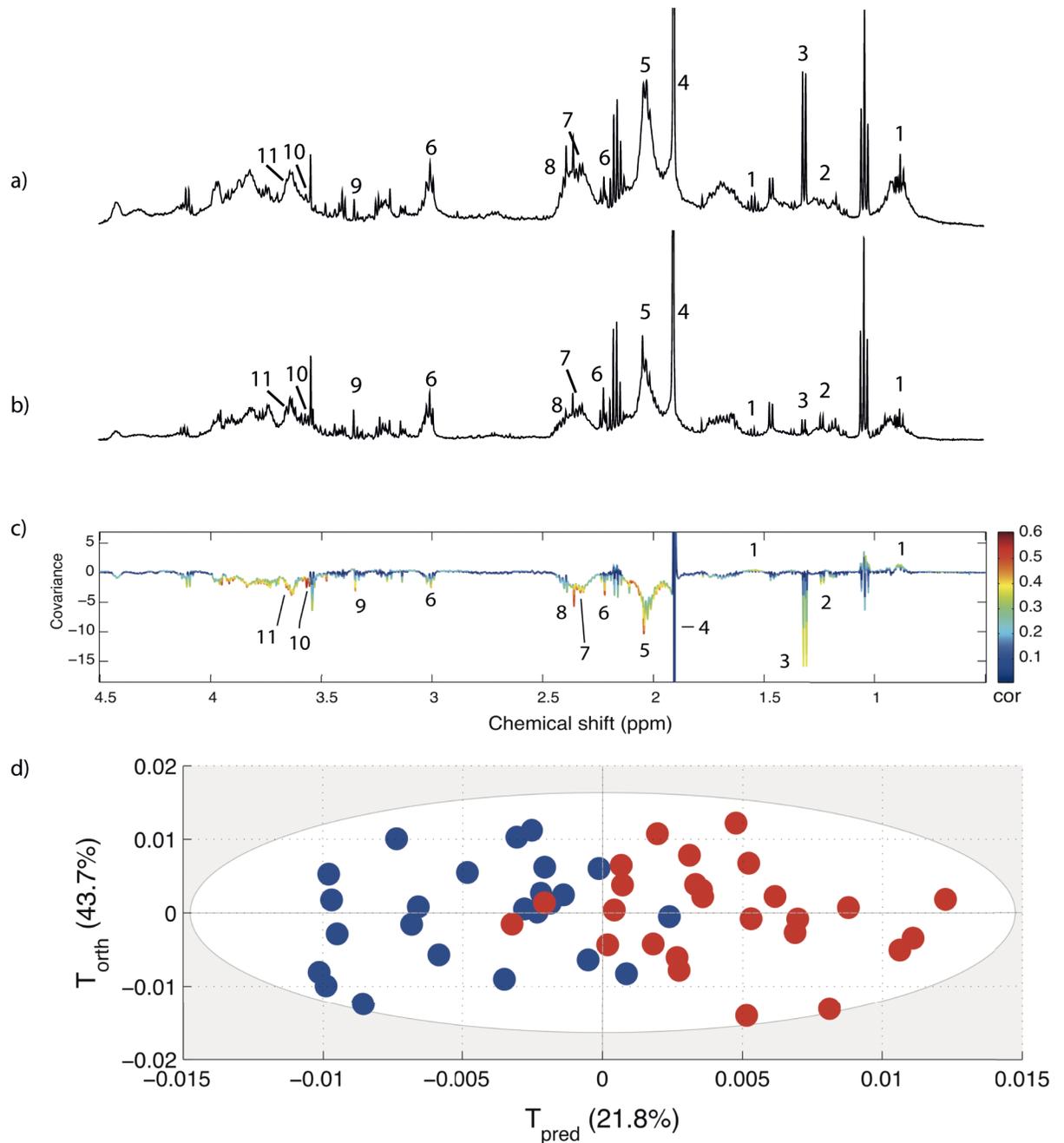
OPLS analysis was performed and showed a good separation between controls <sup>1</sup>H NMR spectra and those of patients (Fig 2c). The quality parameters of the model were R2Y = 0.57 and Q2Y = 0.48.

In this pilot study, metabolomics profiles, interestingly, did not allow us to separate generalized AP from generalized CP as R2, Q2 and CV-AUC were respectively 0.09, -0.62 and 0.2 for one component.

The ability of this model to classify control and patient samples was confirmed by the cross-validation area under the ROC curve (CV-AUROC) of 0.91.

### Identification of metabolites

By analysing the loadings plot, the main metabolites responsible for the discrimination were identified (Fig 2d). Compared to controls, higher concentrations of butyrate (short chain fatty acids) were observed in patients. Conversely, lower concentrations of fucose, lactate, acetate, N-acetyl, gamma-aminobutyrate (GABA), 3-D-hydroxybutyrate, pyruvate, methanol,



**Fig 2. Examples of NMR spectra in saliva and OPLS metabolomic analysis.** a,b) Representative  $^1\text{H}$ -NMR spectra obtained in (a) a control individual and (b) a case individual. c) Orthogonal projection to latent structures (OPLS model) of  $^1\text{H}$ -NMR spectra obtained in saliva from periodontitis patients (red dots) and healthy controls (blue dots) according to the predictive ( $T_{\text{pred}}$ ) and not predictive ( $T_{\text{orth}}$ ) components obtained from the OPLS model. d) OPLS loadings plot showing the discriminant metabolites between patients with periodontitis and controls. Variations of metabolites are represented using a line plot between 0–9 ppm. Positive signals correspond to metabolites present at increased concentrations in the patient group. Negative signals correspond to metabolites present at increased concentrations in the control group. The buckets are labelled according to metabolite assignment (1. butyrate; 2. fucose; 3. lactate; 4. acetate; 5. N-acetyl of glycoprotein; 6. GABA; 7. 3-hydroxybutyrate; 8. pyruvate; 9. methanol; 10. threonine; 11. ethanol).

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**Table 2. Main metabolites identified to discriminate periodontitis from controls according to the loadings plot analysis.** Correlation between bucket intensities and discriminated group members are given with the associated p value.

		Chemical shift (ppm)	Correlation coefficient	p value
1	Butyrate	0.89 and 1.54	0.43	0.001637
2	Fucose	1.23	-0.38	0.005951
3	Lactate	1.31	-0.37	0.007531
4	Acetate	1.91	-0.46	0.000683
5	N-acetyl of glycoprotein	2.04	-0.51	0.000132
6	GABA	2.22 and 3.0	-0.49	0.000263
7	3-Hydroxybutyrate	2.33	-0.49	0.000263
8	Pyruvate	2.36	-0.5	0.000187
9	Methanol	3.35	-0.44	0.001234
10	Threonine	3.56	-0.57	1.3E-05
11	Ethanol	1.20 and 3.65	-0.3 and -0.49	0.032448 and 0.000263

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threonine, ethanol were observed in patients (Table 2) in which the peak number corresponds to the spectral assessment shown in Fig 2d.

### Logistic regression

A univariate analysis was performed as a preliminary step to select the metabolites to be computed for the stepwise regression analysis. Prior to the univariate analysis a binarization of the variables was made by comparison of their respective histograms. Due to the large number of significant variables with regards to the total number of patients (26) and controls (25), we kept the five variables (lactate, GABA, butyrate, threonine, hydroxybutyrate) with  $P \leq 0.2$  (S1 Table in supplementary material). and already identified in the OPLS loadings plot analysis (Table 2) as potentially clinically relevant. Table 3 reports the stepwise logistic regression analysis: 5 metabolites were entered in the model and 3 metabolites (lactate, GABA and butyrate) were recognized as the main potential independent biomarkers for periodontitis diagnosis. The best positive and negative predictive values were those resulted from the computing of the

**Table 3. Multivariate logistic regression analysis.**

Variables	p	OR	95%CI
<i>Initial table</i> <sup>1</sup>			
Lactate	0.0115	13.672	(0.001–44.622)
GABA	0.0328	15.247	(1.249–186.203)
Butyrate	0.1094	7.775	(0.631–95.796)
Threonine	0.1690	1.542 E-134	(0–7.703E56)
Hydroxybutyrate	0.2421	2.67E-14	(4.816E-37–1481137133.037)
<i>Final table</i> <sup>2</sup>			
Lactate	0.0088	13.464	(1.924–94.208)
GABA	0.0043	33.480	(2.999–373.813)
Butyrate	0.0291	14.816	(1.316–166.833)

<sup>1</sup>Analysis of the 5 variables chosen according to the preliminary univariate analysis (see text and supplementary material; analysis done on 51 samples;  $R^2 = 0.394$ );

<sup>2</sup> from the final results of the logistic regression 3 variables were independently associated to periodontal disease (analysis done on the 51 samples;  $R^2 = 0.355$ ).

OR: Odds ratio; CI: confidence interval; GABA: gamma-aminobutyrate.

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**Table 4. Evaluation of the clinical significance of the grouped lactate-GABA-butyrate considered as one.**

	PPV	NPV	Se	Sp
Value (95% CI)	0.77 [0.65–0.88]	0.86 [0.76–0.95]	0.89 [0.80–0.97]	0.72 [0.06–0.84]
sigma	0.06	0.05	0.04	0.06

PPV: positive predictive value; NPV: negative predictive value; Se: sensitivity; Sp: specificity.

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positive and negative predictive values corresponding to all the associations of the values of the three variables of the final table of the multivariate logistic regression: the group lactate = 1-GABA = 1-butyrate = 1 emerged (PPV 0.77) (Table 4).

## Discussion

<sup>1</sup>H NMR spectroscopy is a reliable, cost-effective method for analysis of biofluids [29, 34]. Only a few studies on saliva using this method have been reported, mostly in general diseases, for physiological studies, or for illicit drug uptake [32, 36, 52]. Our group has already investigated human saliva in the context of sarcoidosis [34]. It also must be noted that it has been recently reported that investigating saliva is less influenced by diet than urine [35]. Therefore, this method can be considered as a method of choice for investigating periodontal disease. In the present pilot study, using <sup>1</sup>H NMR spectroscopy, we carried out the metabolic profiling of saliva collected in periodontitis patients before any treatment, and in healthy controls matched for age, gender, and tobacco status.

### Representation of the periodontitis population by this cohort of patients

Clinical data indicated that our sample of 26 patients, aged from 18 to 64, was representative of patients with active periodontitis according to the usual criteria (*i.e.* inflammation, PD, CAL and BL). Cases were recruited according to the periodontal criteria (see [Materials and methods](#)) eliminating stable disease or morphologic abnormalities without inflammatory process (*i.e.* recession).

### Discrimination between controls and patients

Comparison of the <sup>1</sup>H NMR spectra through multivariate statistical analyses showed significant differences between controls and patients in saliva metabolomic profiles. Because of the stringent selection criteria used for patients and controls that excluded most of the confusing bias, it can be considered that disease-specific content was observed in saliva metabolomic evaluations, illustrated by variations of the NMR signal intensity of eleven metabolites when comparing the 2 groups.

Interestingly, in this pilot study, the metabolomic profiles observed in generalized CP and AP were not distinct. Periodontitis, characterized by tissue destruction, is largely considered to be due to an inflammatory-infectious process. Consequently, the metabolomic fingerprints observed in saliva may be considered primarily to be related to tissue destruction mechanisms and, therefore, dependent on the severity of the disease rather than on the type of periodontitis. A second possible explanation suggests that the observed metabolite concentration changes may be related to the products of the pathogenic bacterial population. As the disease progresses, the sub-gingival environment is known to change with regard to oxygen tension, redox potential, pH, and availability of host-derived macromolecules [53–56]. These changes are responsible in a cause-and-effect way for modulation of the composition of the bacterial

community [57]. Metabolomic profiles observed in patients with periodontitis may reflect a disease-associated microbiota, which may share similar metabolite profiles in generalized CP and AP, even if bacterial species found in these clinical entities may be different. It may be hypothesized that the source of the major identified metabolites in oral fluid, allowing us to distinguish patients from controls, is from bacterial populations colonizing the periodontal tissues.

## Metabolic changes in the oral fluid

A metabolomic profile associated with generalized periodontitis was clearly identified in the oral fluid. In particular, a significant increase of butyric acid was found. Some types of anaerobic periodontal bacteria such as *P. gingivalis* and *F. nucleatum*, belonging to *Bacteroidetes* and *Fusobacteria* phyla, produce this metabolite [58–60]. Butyrate, as well as other short chain acids (SCAs), is released into the microenvironment from infection sites and thought to contribute to the pathogenesis of periodontitis through impairment of defence cells or fibroblasts and in epithelial cell functions [58, 60, 61]. Thus, SCAs as butyric acid are found to be associated with periodontal inflammation [62]. In a longitudinal study of patients with chronic periodontitis receiving periodontal treatment, it was reported after chromatography analysis that the concentration of butyric acid in gingival crevicular fluid decreased to reach the same levels found in the healthy control group [62]. However, during the long-term observation period after therapy, gradually increasing concentrations of butyric and isovaleric acids seemed to be associated with recolonization of periodontal pathogens [62]. Pathogenic bacteria are known to be harboured in deep pockets where they find favourable conditions for their development [63]. Increased concentrations of butyrate may indicate a growth in pathogenic subgingival microorganisms linked to the progression of periodontal destruction. On the other hand, in our study, disease-specific increases of butyrate concentrations are associated with reduced levels of other SCAs such as lactic acid, acetic acid, formic acid, or  $\gamma$ -aminobutyric acid. The marked reduction of lactate in patients could reflect a shift in the microbial composition of commensal bacteria in the healthy oral cavity as well as in other mucosal ecological niches. This may include lactic acid bacteria [64], producers of lactic acid [65, 66]. In mucosal compartments, these organisms are known to contribute to the control of microbiota by competing with other microorganisms for adherence to epithelial cells and by producing antimicrobial compounds [65, 67]. This symbiotic relationship includes a complex molecular cross-talk between LAB and the host [68]. For instance LAB species can modulate immunological functions in the digestive tract, such as the enhancement of the ratio between anti-inflammatory (IL-10, TGF $\beta$ ) and pro-inflammatory (IL-1 $\beta$ , IL-3, IL-4) cytokines [69]. On the other hand, the immune system selects the LAB species to be accepted. Therefore, vaginal microbiota of healthy reproductive women are often dominated by LAB species, contributing to the maintenance of good health by producing acid, hydrogen peroxide, and bacteriocin-like substances [65, 70]. Lactic acid is able to inhibit the growth of many bacteria, particularly Gram-negative species, by disrupting their outer membrane [71, 72]. With this ability to produce substantial amounts of lactic acid, oral LAB species might be recognized as beneficial species for the microenvironment between the teeth and the gum by inhibiting the growth and preventing the colonization of exogenous Gram negative pathogens [73], even if carbohydrate fermentation acid end products of lactobacilli or streptococci are responsible for dental caries formation [74]. Metabolomic profiles showing low levels of lactic acid in patients compared to controls could reflect the antagonistic activities between strains of lactic acid bacteria and Gram negative periodontal pathogens like *P. gingivalis*, *F. nucleatum*, and *T. denticola* [75]. The observed decrease of gamma-aminobutyrate (GABA) in the oral fluid of patients corresponds as well

with a reduced population of LAB. Indeed, some LAB species biosynthesize GABA by glutamate decarboxylation. In addition, logistic regression showed that the combination of three metabolites (lactate, GABA, butyrate) present in proportion to their respective thresholds is associated with a high risk for periodontal disease activity (VPP of 0.77).

## Conclusion

In summary, the present study designed as a pilot study using  $^1\text{H}$  NMR spectroscopy analysis of saliva in periodontitis has shown its ability to identify clinically relevant biomarkers. It must be stressed that  $^1\text{H}$ -NMR while a fast, robust, quite simple and reproducible method for small metabolites analysis has been exceptionally tested for periodontitis on a clinical scale. It can be largely expected that, after a validation step on a larger cohort of patients,  $^1\text{H}$ NMR analysis of saliva may offer a considerable help in the early diagnosis and follow-up of periodontitis. The metabolomics signature obtained by  $^1\text{H}$  NMR suggests that the main signals identifying a periodontitis-related oral fluid milieu derive from bacterial metabolism. An alternative approach focused on the salivary microbiota in patients with periodontitis has been recently published [76]. While the method is a less easy one and completely different it would be of great interest to share the data of both methods on the same patients as dysbiosis is considered the main etiologic factor and metabolomics reflects small metabolites from the microbiota as well as from the inflammatory reaction of the host. In conclusion metabolomic profiles most probably in line with a shift in oral microbiota are a potential tool of choice for diagnosis, management, and follow-up of patients. Indeed, it needs before generalisation of the method a validation step on a larger cohort of patients and controls.

## Supporting information

**S1 Table. S1 Table of the univariate analysis (Mann-Whitney test) of the metabolites identified by the OPLS loadings plot as relevant.** NMR data are available on metabolight platform as EMTBLS524.  
(DOCX)

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