GINGIVAL TISSUE METABOLITES AS BIOMARKERS OF PERIODONTAL DISEASE: A PILOT STUDY

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BACKGROUND

INTRODUCTION

Periodontitis is a chronic multifactorial inflammatory disease associated with dysbiotic plaque biofilms leading to progressive breakdown of the periodontal tissues. The main characteristics of periodontitis include the loss of periodontal tissue support along with signs of inflammation such as clinical attachment level (CAL) loss, radiographic bone loss (RBL), increased periodontal probing depth (PD) and bleeding on probing (BoP). Periodontal disease is a public health problem with high prevalence and it also affects people's lives due to a negative impact on systemic health, chewing function as well as aesthetics.[1–3]

In 2010, severe periodontitis was the 6th most prevalent disease worldwide with a prevalence of 11.2%.[4] The prevalence showed to rise with age, especially between 30 and 40 years of age with a peak around 38 years. These data are confirmed by a study conducted between 2009 and 2014, which estimated that 42% of US adults ≥30 years old had periodontal disease, 7.8% of those having severe periodontitis.[5]

Moreover, the study design of national surveys of periodontitis uses partial-mouth exam protocols.[6] Studies showed that partial examination underestimate the prevalence of disease, and the variance of underestimation depends on the number and sites examined. When the estimates were adjusted for the bias, among the United States population aged ≥30 years the total prevalence percentage of subjects with periodontal disease was 48.2%.

Despite this high prevalence and the increased knowledge of the etiology of periodontal disease, the diagnosis is still based on traditional clinical assessments.[7] To

make a periodontal diagnosis the clinician must evaluate the presence or absence of clinical signs of inflammation (i.e. BoP; PDs; extent and pattern of CAL loss and RBL); patient's medical and dental histories; presence or absence of signs and symptoms, including pain, and amount of detectable plaque and calculus.[1,2] Furthermore, development of an accurate system that indicates prognosis is an essential component of the treatment plan. However, evidence-based prognostic predictors for periodontitis are lacking.[8]

The periodontal probe is still considered the instrument of choice for assessing the periodontal status at the time of the examination. However, there are many factors that influence the measurements taken with the periodontal probe and the ability of the probe to accurately measure the depth of the pockets has been questioned.[9]

The reproducibility of PD and CAL measurements have been investigated, and it has been concluded that there is a deviation of 1mm in repeated measurements.[10] Probing force also had a significant effect of the reproducibility of PD measurements.[11,12] In addition, since different periodontal probes exist, the probing force should be determined based on the probe tip diameter.[13]

Bleeding on probing is still an integral part of a periodontal exam. Studies have demonstrated the value of lack of BoP as a predictor for periodontal stability, whereas the presence of BoP was not a good predictor for disease progression.[14] Several indices have been proposed to measure and assess the gingival inflammatory status, but there is still not accepted level for prevalence of BoP above which the risk of disease recurrence has been established.[15]

Overall, our current methods of assessing periodontitis identifies history of disease but unfortunately is unable to reliably predict future disease. Although we still rely on our CAL measurements to formulate a diagnosis, newer approaches are being

investigated in order to predict rather than making a diagnosis based on what has already occurred. Therefore, the research is now focused on identifying genetic, microbial, and host response-associated markers that not only distinguish different periodontal conditions but may also predict the onset and progression of periodontal disease before the periodontal tissues are severely damaged.

GENETIC MARKERS

With increasing knowledge of the genome, it is apparent that there is a genetic basis for most diseases, including periodontitis.[16] This fact has reinforced the idea that we may use genetic tests to evaluate disease risk and to develop treatments accordingly. However, we are still far from determining specific genetic profiles for both aggressive and chronic periodontitis.

Data from pairs of adult twins clearly indicate that periodontitis has a substantial heritable component.[17] Of the periodontal diseases, the genetic or heritable predisposition seems to be more associated with aggressive periodontitis. Inherited phagocytic cell deficiencies, abnormalities of leukocyte chemotaxis and adhesion appear to confer risk for aggressive forms of periodontitis.[18–20]

Whether or not genetic factors influence the more common adult chronic periodontitis is less clear.[21]

The real challenges to developing diagnostic tests for periodontitis are due to genetic polymorphisms which have also been associated with the susceptibility of the disease.[22] Genome studies and screening of nucleotide polymorphisms have provided new data, but without a definitive solution for the treatment and management of periodontitis.[23]

Researchers have also looked at gene expression of subjects with chronic or aggressive periodontitis.[24] Gene-expression profiling (i.e. the systematic cataloguing of mRNA sequences in a cell population or tissue sample) is a genomic tool that may provide a new approach to study the pathophysiology of periodontitis.[25] The main goal is to understand whether gingival tissue transcriptomes may serve as the basis for an alternative classification of periodontitis.[26] In addition, gingival tissue transcriptomes may be a viable instrument for the early detection of disease progression, which can be clinically relevant to improve monitoring the disease. However, the limitation of the transcriptome is that not all transcripts are translated into proteins because of post-transcriptional modifications and degradation. Proteomics has similar problematic issues because even though certain proteins or enzymes may be present there is no way to establish if they perform the functions that they can do. On the other hand, metabolomics is the result of (i) viable transcripts that are translated into proteins which (ii) are functioning at metabolizing substrates into products.

BIOMARKERS IN THE GINGIVAL CREVICULAR FLUID

Gingival Crevicular Fluid as Diagnostic Biomarker for Periodontitis

Gingival crevicular fluid (GCF) is a serum transudate or inflammatory exudate that originates from the periodontal tissues and can be harvested from the gingival sulci.[27] Being non-invasive, GCF may be utilized as diagnostic fluid for periodontal diseases.[28–30] Overall, the proteomic science provides evidence of healthy and diseased status analysing protein compositions.

Human GCF was discovered in 1952, and its oral defence mechanism was demonstrated in the late 50's.[31,32] Gingival crevicular fluid contains local breakdown

products such as inflammatory mediators, serum transudate, subgingival microbial plaque, extracellular proteins, and cells. The protein composition of GCF may represent the pathobiology of periodontitis, as its composition varies between healthy and diseased conditions.[33,34]

Several inflammatory mediators have been identified from GCF, including cytokines, proteinase and proteins.[35] These factors are promising to be possible diagnostic markers of periodontitis.[36] Gingivitis, healthy sites and periodontitis sites may be distinguished with an MMP-8 test.[37] Other frequently reported biomarkers associated with periodontitis in the GCF are interleukin-1β (IL-1β) and receptor activated nuclear factor-kappa B ligand (RANKL).[38]

Recently, the use of highly sensitive mass spectrometric technology has allowed the detection of proteins of many biological specimens, including the GCF.[39–41] The GCF proteome from healthy, gingivitis and periodontitis sites was found different, and its analysis may provide us valuable information on the periodontal pathogenesis.[42] The protein composition of GCF may represent the pathophysiology of periodontal disease; protein profiles of GCF collected from healthy sites are being investigated as GCF proteomic patterns, potentially to be used as a tool for identification of biomarkers of periodontitis by proteome analysis. Smith is the one who started to look into GCF proteomics in a site-specific way. However, proteomic analysis of GCF is still at an early stage with limited evidence in the literature.[33]

However, a final piece of the omics puzzle is still missing: the metabolomics.[43] Metabolomics offers unique insights into small molecule regulation and signalling in biology which cannot, otherwise, be discovered by the proteomics and genomics approaches.[44] Metabolomics is already the new frontier of biologic research, similar to proteomics, transcriptomics and genomics.[45] Metabolomics gives us a screenshot of

the metabolic activity that represents the response of organic systems to pathophysiological stimuli.

The two major technologies used to identify metabolites are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. The main advantage of MS is sensitivity, which allows the test to detect a large number of metabolites. This allows the measurement of hundreds of individual species of metabolites within a single sample, with only few false negative results. MS however require significant pre-analysis processing with derivatization of the chemical species in the sample. The major weaknesses of MS are the major strengths of NMR spectroscopy. Nuclear magnetic resonance requires minimal pre-analysis processing of the samples and yields very precise and less biased outputs; the peak area of a compound in the NMR spectrum corresponds to the concentration of specific nuclei (i.e. ^{1}H, ^{13}C), making the identifications and quantification of metabolites very precise. Preliminary data are shown by a comparison between deep periodontal pockets with healthy sites, metabolites revealed in the GCF through MS were quantified and the resulting spectra were evaluated.[46] Nineteen metabolites were identified and their levels were associated to the clinical status (diseased versus healthy) of the periodontal tissues. The authors concluded that metabolic analysis of GCF through MS may a valuable tool for the discovery of diagnostic biomarkers of periodontal disease. However, although data from MS studies are encouraging, investigations using NMR are still lacking in the literature. Results from both MS and NMR will allow for further identification of metabolites and understanding the pathophysiology of periodontitis.

Gingival Crevicular Fluid as Prognostic Biomarker for Periodontitis

GCF not only can be a future diagnostic instrument, but it may also aid in the detection of the progression of periodontitis. Increased levels of active collagenase-2, matrix metalloproteinase-8, (MMP-8) in the GCF were associated with progression of periodontitis.[47] IL-1β , RANKL and MMP-8 have all been established as indicators of alveolar bone resorption, and they have also shown positive correlation with disease progression.[48–52] A positive correlation between GCF levels of IL-1β, IL-8 and MMP-8 and clinical signs of periodontitis was also demonstrated.[53] Therefore, the analysis of biomarkers in GCF may be helpful in forecasting patient vulnerability to future attachment loss.[54]

Findings through metabolomics should also provide new insights of biomarkers for periodontal disease progression. Researchers have tried to identify metabolites of GCF obtained from healthy, gingivitis, and periodontitis sites in humans.[55] The purine degradation pathway, which is integral part of the reactive oxygen species (ROS) production, has been found significantly abundant at a disease status. This may prove that the oxidative stress and inflammation may be mediated through this pathway in periodontitis sites. In this view, metabolomic analysis of GCF could be useful even for prediction and not only diagnosis of periodontal disease.

In addition, to clarify the role of metabolic profiles in aggressive periodontitis, identification of the differential metabolic profiling between patients with generalized aggressive periodontitis (GAgP) and healthy controls has been performed.[56] Serum and GCF metabolites were significantly different between sites of GAgP and healthy periodontium. Therefore, these metabolic profiles have great potential in detecting GAgP and elucidating its pathophysiology.

MARKERS IN THE GINGIVAL TISSUE

Detection of metabolites may be carried out not only in cells and biofluids, but also in tissues by either NMR or MS. In particular, NMR technology has been increasingly used in the field of medicine to analyse the metabolites present within the tissues.[57-60] The main goal is to integrate different metabolomics approaches in the field of basic and biomedical sciences, and try to enrich our knowledge of cellular and physiological biology, especially looking at metabolic pathways associated with disease.

Although the progress of the metabolomics world has just started, several medical applications have already been suggested. For instance, a potential for the metabolomics has been demonstrated to be valuable in oncology, including early detection and diagnosis of cancer.[57] Brain tumor biopsy studies have been conducted, which should enable the advent of new clinical instruments to better evaluate operable cancers via tissue molecular identification.[58] Less invasive treatments can also be performed thanks to the metabolomics. For example, the metabolic profile of gall bladder tissues can be used rather than extraction procedures which are time consuming, laborious and increase the risk of contamination.[59]

Histopathological analysis of breast tumor specimens is, along with lymph node status, the basis for a treatment plan. However, evaluation of intact breast tissue samples is a rapid method, providing spectra with resolution where relative quantification of the majority of the detected metabolites is possible.[60]

The combination of human biomarkers and bacterial biomarkers needs to be further investigated, taking into consideration also changes occurring before, during and after periodontal treatment. Gingival tissue biomarkers for periodontal disease have not been investigated yet, but they may allow the distinction between patients with

different periodontal diseases and healthy individuals. Therefore, gingival tissue metabolites, in the same way of GCF metabolites, could potentially be applied in clinical settings to evaluate or monitor the periodontal status of patients. This could allow us to make an early diagnosis and predict the progression of periodontal disease.

MISSING INFORMATION

- Do the changes of metabolites in the periodontal soft tissue correlate to the ones in the GCF when comparing healthy and post-S/RP periodontitis sites?

- Are the metabolites present in the periodontal soft tissues different between healthy and periodontitis subjects after initial periodontal therapy?

HYPOTHESIS

- Metabolites in the gingival tissue are correlated and representative of metabolites in the GCF and capable of discriminating between healthy and post-S/RP periodontitis sites.

SPECIFIC AIMS

- Determine the correlation between the gingival tissue metabolites and the metabolites in GCF identified in healthy and in post-S/RP periodontitis sites.

- Map the metabolites in the gingival tissues of periodontal healthy sites and of periodontitis after non-surgical periodontal therapy (SRP) using proton nuclear magnetic resonance (H-NMR) spectroscopy.

GINGIVAL TISSUE METABOLITES AS BIOMARKERS OF PERIODONTAL DISEASE: A PILOT STUDY

INTRODUCTION

Periodontitis is defined as a chronic multifactorial inflammatory disease associated with dysbiotic plaque biofilms leading to progressive breakdown of the tooth-supporting tissues.[1] Despite the high prevalence and increased understanding of the pathobiology of periodontal disease, the diagnosis is still based on traditional clinical assessments.[2] The main characteristics of periodontitis include the loss of periodontal tissue support along with signs of inflammation such as clinical attachment level (CAL) loss, radiographic bone loss (RBL), increased periodontal probing depth (PD) and bleeding on probing (BoP).[3,4]

The periodontal probe is still considered the instrument of choice to diagnose periodontitis in clinical practice. The probe, however, is capable of assessing only past history of periodontal disease but unable to predict disease initiation or progression of existing disease. In addition, the ability of the probe to accurately measure the depth of the pockets has been questioned.[5-8] The efforts to identify predictors of future disease have been relatively slow and hindered by tests with low sensitivity and specificity.

The gap in the knowledge resides in the identification of specific biomarkers that can identify the initiation and progression of periodontitis. Some evidence showed that metabolite composition of gingival crevicular fluid (GCF) may represent the pathobiology of periodontitis, as the GCF contents differ between healthy and diseased sites.[9] Therefore, being non-invasive, GCF has drawn the attention of researcher and may be used as diagnostic fluid for periodontal diseases assessment.[10-12]

On the other hand, gingival tissue biomarkers for periodontal disease have not been investigated yet, but they also may allow the distinction between subjects with different periodontal diseases and healthy individuals. Furthermore, it is critical to establish the correlation between GCF metabolites and metabolites within the gingival tissue in order to use the GCF as a diagnostic tool for the detection of periodontal disease.

This cross-sectional pilot study aims at testing the correlation between the metabolites identified in gingival tissues and the ones revealed in GCFs in healthy sites and in periodontitis sites after S/RP. The secondary aim is to map the metabolites in the periodontal soft tissues that are capable of discriminating between periodontal health and periodontitis after scaling and root planing (S/RP).

MATERIAL AND METHODS

Subject Recruitment and Inclusion/Exclusion Criteria

The pilot study was approved by the Institutional Review Board (IRB) of the University of Minnesota. Sixteen subjects were recruited from the Division of Periodontology of the University of Minnesota School of Dentistry, signed consent as well as HIPPA form (Health Insurance Probability and Accountability Act, 1996) were obtained from all participants prior to enrolment. Nine subjects presenting stage III or IV periodontitis were grouped as “periodontitis”, whereas 7 subjects with healthy periodontium were grouped as “non-periodontitis”.

The inclusion criteria for the periodontitis subjects were: diagnosis of stage III or IV periodontitis with ≥5 teeth presenting CAL ≥5 mm, at least one site with PD ≥7 mm and more than 33% of RBL detectable on the full mouth radiographic examination. The

non-periodontitis subjects recruited presented no history or presence of periodontal disease and were referred for elective crown lengthening procedure. All subjects with uncontrolled systemic diseases were excluded from the study as well as subjects who had antibiotic therapy in the last three months. All study subjects received an initial comprehensive periodontal exam which included the clinical measurements such as PD, CAL, plaque control (PC), BoP, furcation involvement and tooth mobility.[13-16]

Sample Collection

After initial exam, the periodontitis subjects received full mouth S/RP, a periodontal re-evaluation at 6-8 weeks and periodontal surgery performed in sites with residual PDs ≥5 mm. On the day of the periodontal surgery after S/RP, GCF samples were harvested before anesthesia from the same sites selected for gingival biopsy for comparison. The following steps were taken in order to collect the GCF systematically in each site: 1) proper isolation from salivary contamination by means of a cotton roll; 2)light jet of air directed 45^{o} angle from the sulcus towards the coronal aspect of the tooth; 3)supragingival plaque was removed with a curette; 4)5-micron porous silver membrane 3.96 mm of diameter was inserted into the gingival sulcus and kept for 30 seconds; 5)the membrane was placed dry in an Eppendorf tube and stored at -80^{o}C. Following GCF sampling gingival tissue samples were harvested from each subject utilizing the secondary flap of the deepest interproximal pocket involved, the gingival tissue was excised by means of a 2x2 mm tissue punch. Gingival samples were stored at -10^{o}C in dedicated plastic tubes until laboratory processing.

The non-periodontitis subjects underwent crown lengthening procedure and the secondary flap tissues form an interproximal site were used to obtain gingival tissue

samples as described for the periodontitis subjects. The day of the crown lengthening procedure, GCF samples were also obtained before anesthesia from the same sites selected for gingival biopsy with the same above-mentioned steps for the periodontitis subjects.

Sample Preparation/Processing

Gingival tissues

Twenty-four hours prior to laboratory process, the gingival samples were thawed and a solution with deuterium water (D_2O), 2,2-Dimethyl-2-silapentane-5-sulfonic acid (DSS), phosphate-buffered saline (PBS) and sodium Azide (NaN_3) was added into the sample tubes.[17] Gingival biopsies were kept at +4ºC for 24 hours and then run with High-Resolution Magic-Angle-Spinning (HR-MAS) proton Nuclear-Magnetic-Resonance (H^+-NMR) spectroscopy.[17]

Gingival crevicular fluid

Fluid tissue extract protocol for the GCF samples before laboratory process included: 1) thawing of the membranes; 2) adding 40 µL of H_2O-Mass Spectrometry Grade water; 3) vortexing and centrifuging at 13,000 rpm for 10 minutes; 4) separation of the 40 µL aliquot from the silver membrane by placing the solution in another Eppendorf tube; 5) adding 10 µL of D2O (deuterium water) + 2,2-Dimethyl-2-silapentane-5-sulfonic acid (DSS) [5x concentrated] + PBS [5x concentrated] + AZIDE [5x concentrated] to the 40 µL aliquot solution sample; 6) freeze the Eppendorf tube again to -80ºC.[18] Twenty-four hours prior to proton NMR, all the tubes containing the GCF samples were thawed and the solution transferred to individual 1.7 x 103.5mm borosilicate tubes (SampleJet [cat#Z106462] – Bruker – Germany) utilizing long gel-

loading pipet tips (Sorenson [cat#13810]) and refrigerated at +4ºC until NMR analyses 24 h later at the University of Minnesota NMR Center.

NMR Analysis

The metabolic profiling in solid tissues is obtained with a rapid spinning of a sample at a magic angle (54.7º) that gives high resolution hydrogen (H^+) spectra under an induced magnetic field. The samples are placed in a standard rotor insert (50 µl volume size) and spin at 4-6 kHz at +25ºC for 40 minutes. Spectral frequencies are captured and matched with Chenomx software to known metabolites libraries and to the Human Metabolome Database (HMDB) libraries for metabolite identification.[19]

On the other hand, GCFs are run with H^+-NMR settings for biological fluids. This technology allows to capture and transform molecular spectra from the samples in frequencies. Those frequencies are then matched against spectral libraries in order to infer compound structures and identify specific metabolites.

Coronavirus (Sars-CoV-2, COVID-19) Pandemic

Due to a pandemic severe acute respiratory illness caused by a novel coronavirus (Sars-CoV-2, COVID-19) first detected in Wuhan (Hubei Province, People's Republic of China) in December 2019, the Centers for Disease Control and Prevention (CDC) had imposed strict restrictions which prevented the NMR laboratory to repair a malfunction of the machine for about 1-year time (from March 2020 to April 2021). This partially compromised this pilot study because only the gingival tissue samples had been analysed through HR-MAS proton NMR before the pandemic restrictions. However, we were able to obtain GCF data from a parallel longitudinal ongoing study

investigating on the GCF composition changes between non-periodontitis and periodontitis sites after S/RP with the same inclusion and exclusion criteria as well as material and methods of this study. Gingival crevicular fluid data were used for comparison with our gingival tissue data. The GCF samples were collected from 21 healthy sites from 5 non-periodontitis subjects and 21 post-S/RP diseased sites from 7 periodontitis subjects.

Statistical Analysis

Although the Chenomx software is able to calculate the concentration of metabolites in μM based on the concentration of the DSS standard solution, the amount of each gingival tissue sample and the relative solution were unknown to quantify the metabolites identified through HR-MAS H^+-NMR spectroscopy. However, to quantify relatively the levels of the metabolites a normalization of the μM values to the mean concentration of the metabolites in each sample was performed. The values reported are unitless, calculated from the equation:

$$\text{Relative concentration} = \frac{\text{(Concentration of single metabolite, μM)}}{\text{(Average concentration of all metabolites in the sample, μM).}}$$

Data were submitted to MetaboAnalyst 4.0 (Wishart Research Group, U Alberta, Canada: www.metaboanalyst.ca) with a normalization to the median and 25% range interquartile. Principal component analysis (PCA), partial least squares discriminative analysis (PLS-DA) for 2D scores plot and variable importance in projection (VIP) were performed. A Spearman's correlation test was performed to evaluate the relationship between the changes in metabolites found in the gingival samples of this study and the changes revealed in the GCFs of the parallel investigation. The comparison was

conducted centering and scaling each metabolite to have a mean 0 and standard deviation (SD) 1; mixed effects linear models for each metabolite were used with fixed effects for disease status and random effects for individuals to account for multiple sites within individuals.

RESULTS

Sixteen subjects were recruited in the study, 6 females and 10 males aged 26-84. Nine subjects had periodontal disease with stage III or IV (periodontitis subjects) and had been treated with S/RP, whereas 7 had a healthy periodontium (non-periodontitis subjects).

Gingival biopsies were harvested from each subject and a total of 27 metabolites were identified and quantified: Alanine, Arginine, Asparagine, Aspartate, Choline, Glucose, Glutamate, Glutamine, Glycerol, Glycine, Histidine, Hypoxanthine, Isoleucine, Lactate, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Taurine, Threonine, Tryptophan, Tyrosine, Uracil, Valine, π-Methylhistidine.

PCA and PLS-DA scores plots (Fig. 1a,b) displayed significant clustering of the sample sets, with healthy sites (non-periodontitis subjects) shown in green and samples from post-S/RP periodontitis sites (periodontitis subjects) in red. When considering the periodontitis and healthy sites as groups, the data showed a variance of 32.3% according to the PLS-DA.

PCA

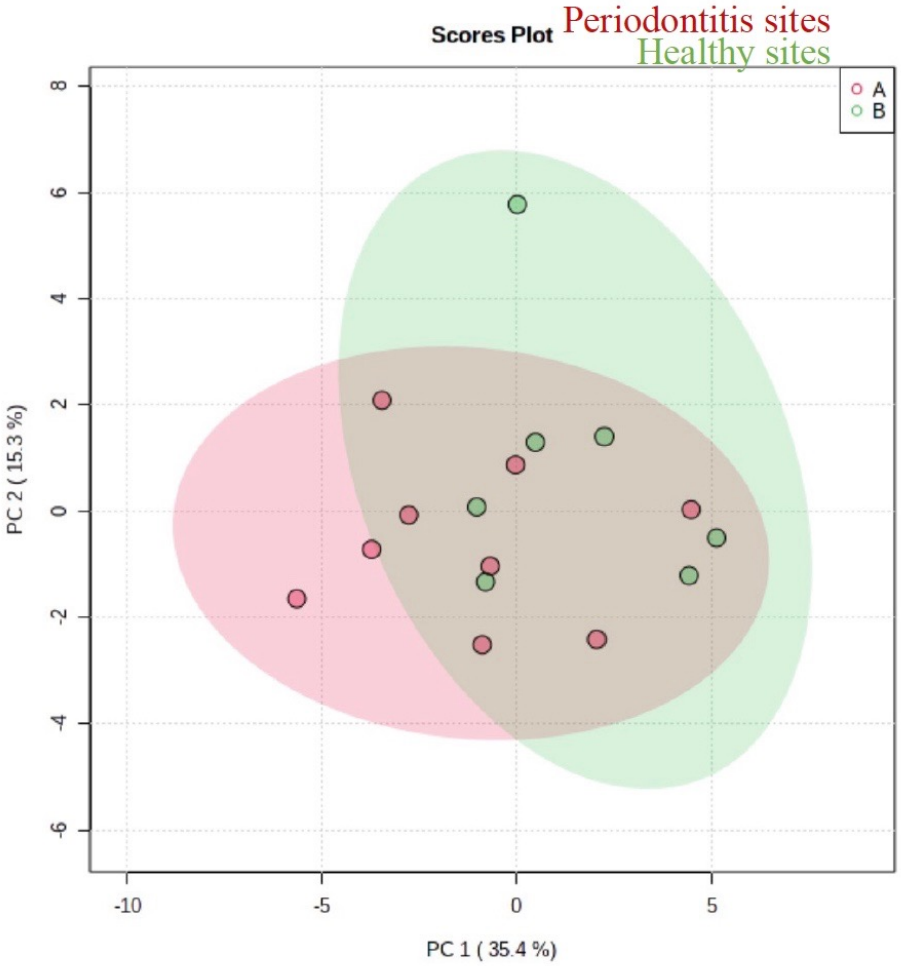

FIGURE 1a Principal component analysis (PCA) for 2D scores plot; periodontitis sites are red versus healthy sites in green.

PLS-DA

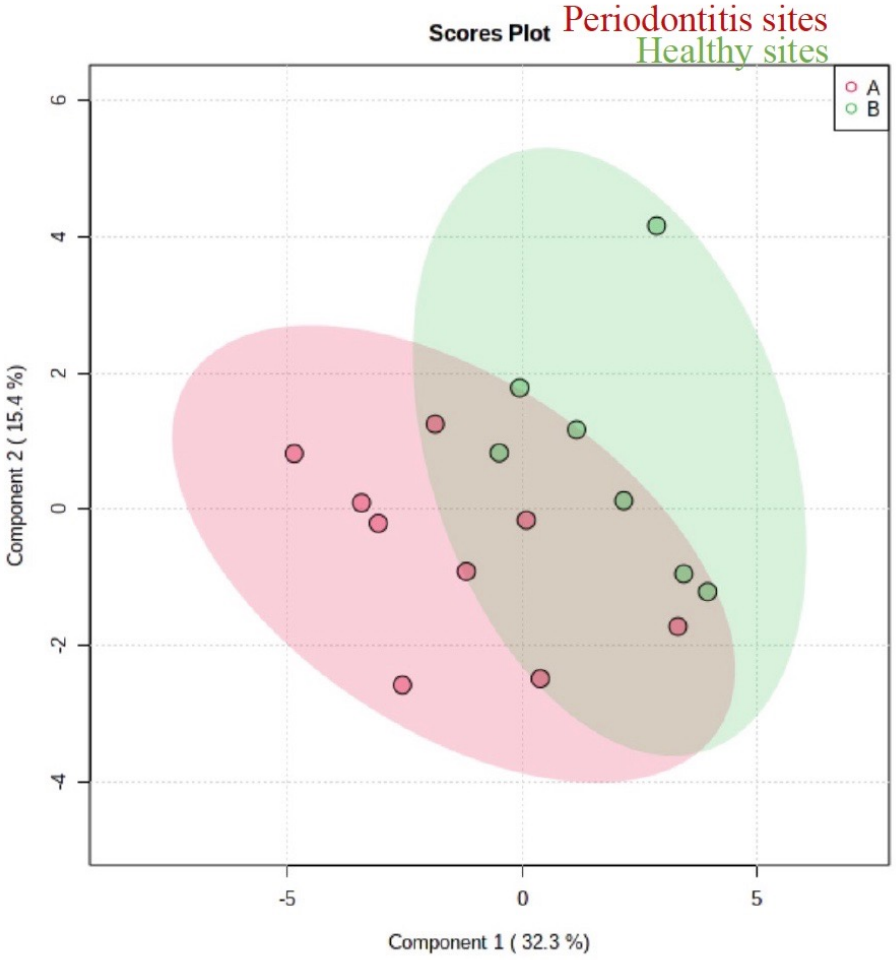

FIGURE 1b Partial least squares discriminative analysis (PLS-DA) for 2D scores plot; periodontitis sites are red versus healthy sites in green.

Although unpaired t-test failed to prove statistically significant difference between all identified metabolites of post-S/RP periodontitis and healthy sites, Aspartate showed a VIP score above the “1.5” which demonstrated a trend in projection for statistical significance (Fig. 2). It is noted that Aspartate was found in greater amount in the clinically inflamed periodontitis sites after S/RP compared to the healthy ones.

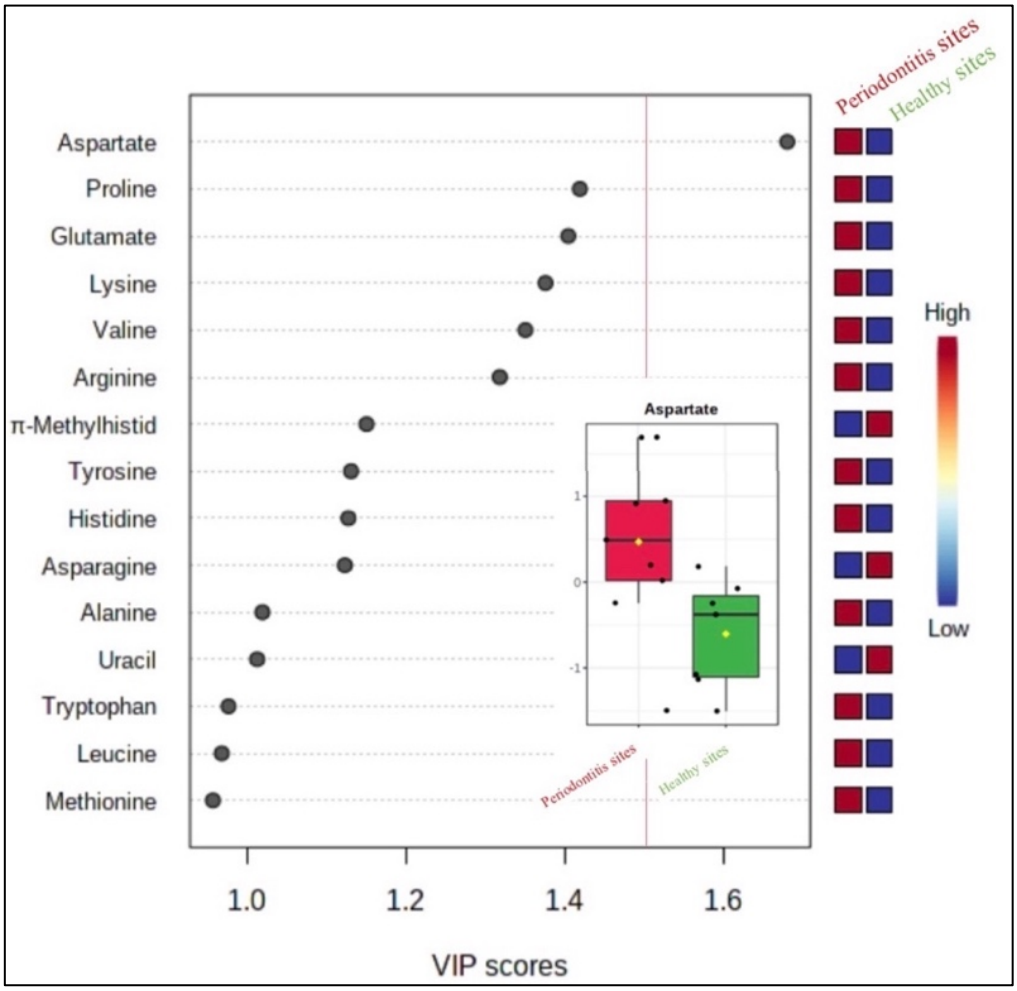

FIGURE 2 Variable importance in projection (VIP) scores; periodontitis sites are red versus healthy sites in green.

GCF data from a parallel longitudinal ongoing study were obtained for comparison with our gingival tissue data. The GCF samples were collected from 21 healthy sites from 5 non-periodontitis subjects and 21 post-S/RP diseased sites from 7 periodontitis subjects. Descriptive statistics of the data set used is illustrated in Table 1. Comparing the changes of metabolites in healthy and post-S/RP periodontitis sites (Fig. 3) between the gingival samples of this study and the GCF data of the parallel investigation, a Spearman's correlation test (Fig. 4) demonstrated a positive correlation ($r=+0.41$) approaching statistical significance (p-value=0.07).

#	GCF healthy sites		GCF post-S/RP periodontitis sites		Solid tissue healthy sites		Solid tissue post-S/RP periodontitis sites	
	Tooth	PD	Tooth	PD	Tooth	PD	Tooth	PD
1	3 (mB)	2	29 (mB)	6	3 (mB)	3	18 (mL)	5
2	9 (mB)	3	28 (dB)	5	3 (dB)	2	19 (dB)	6
3	25 (dB)	2	19 (mB)	5	14 (mB)	1	18 (dB)	7
4	28 (mB)	3	14 (mB)	5	22 (mB)	1	15 (mB)	5
5	7 (mB)	2	8 (mB)	5	24 (dB)	3	21 (dB)	5
6	9 (mB)	2	19 (mB)	6	25 (dB)	2	2 (dB)	7
7	25 (mB)	1	12 (P)	7	27 (mB)	1	18 (mB)	7
8	28 (mB)	2	14 (dP)	8			2 (mB)	8
9	5 (mB)	3	2 (dP)	8			24 (dB)	6
10	21 (mB)	2	9 (mB)	7				
11	9 (mB)	1	27 (mB)	6				
12	30 (mB)	2	19 (mB)	7				
13	9 (mB)	2	31 (mB)	6				
14	12 (mB)	3	13 (dB)	5				
15	25 (mB)	2	3 (mP)	6				
16	28 (mB)	3	25 (dB)	7				
17	31 (mB)	3	30 (dB)	5				
18	22 (mB)	2	3 (mB)	6				
19	3 (mB)	3	25 (mB)	5				
20	9 (mB)	2	19 (mB)	6				
21	20 (mB)	3	20 (mB)	5				
Mean ± SD	**N/A**	**2.27 ±0.64**	**N/A**	**6.00 ±1.00**	**N/A**	**1.86 ±0.90**	**N/A**	**6.22 ±1.16**

TABLE 1 Descriptive statistics of the data set. # = number of sample; GCF = gingival crevicular fluid; S/RP = scaling/root planing; PD = probing depth.

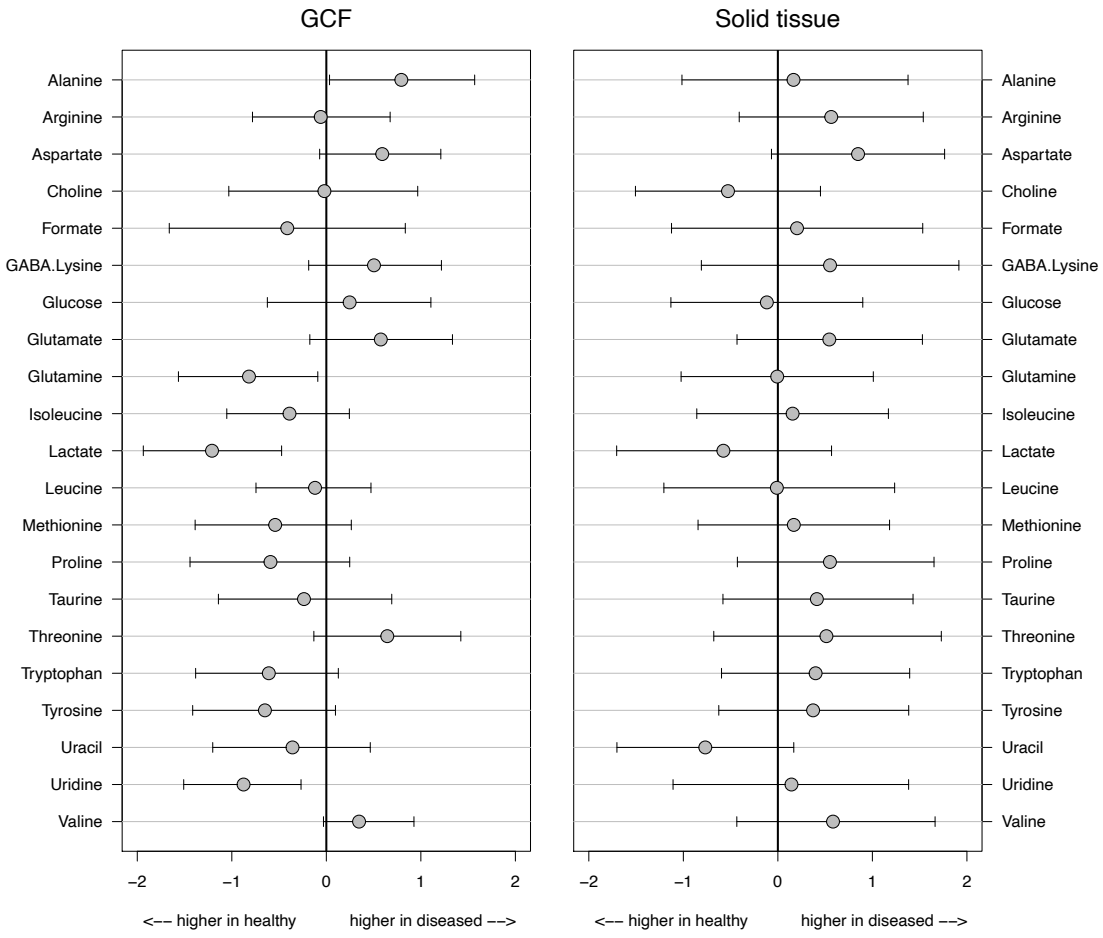

FIGURE 3 Differences in metabolite levels between healthy and post-S/RP sites for gingival crevicular fluid (GCF) and periodontal soft tissue (solid tissue).

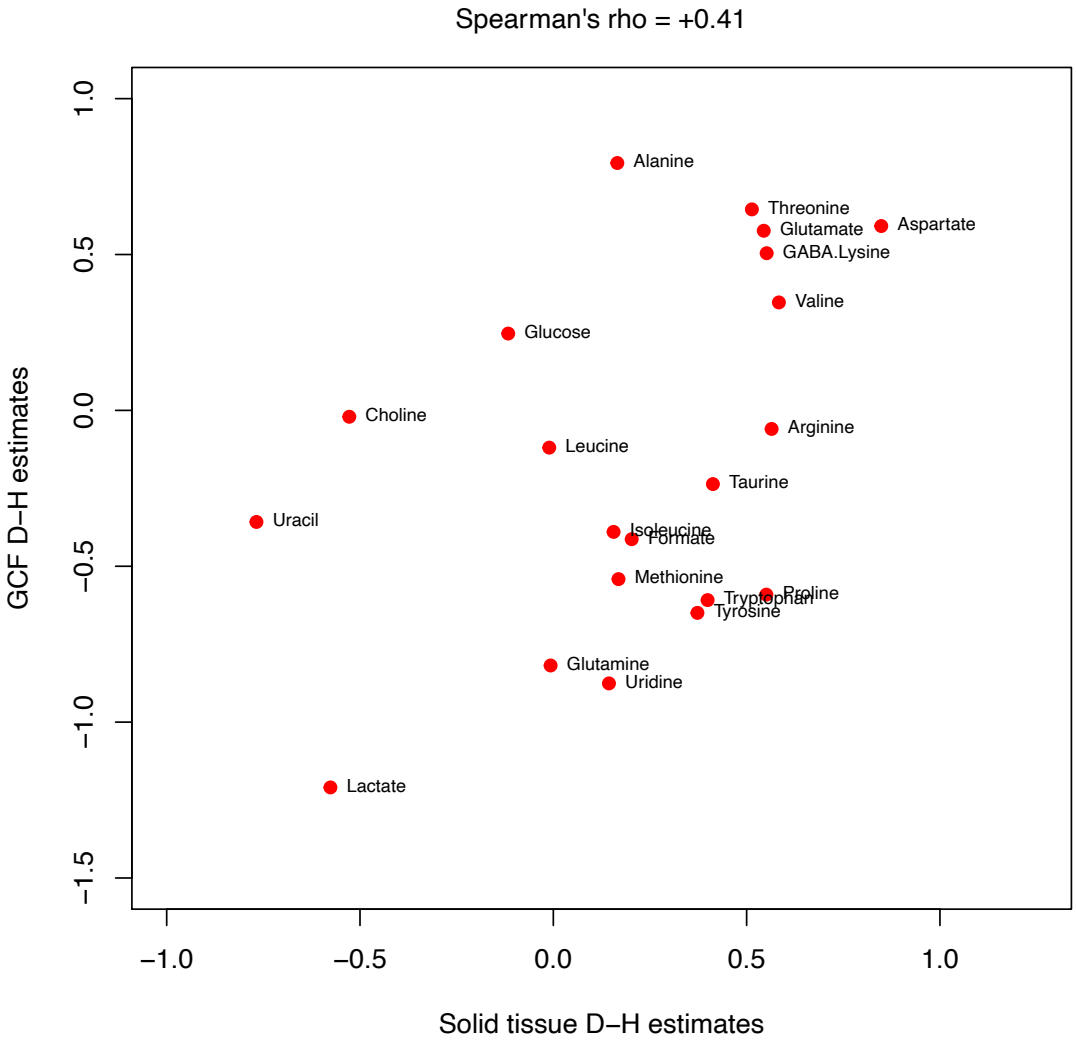

FIGURE 4 Spearman's correlation test scatterplot showing a positive correlation (rho=+0.41; p=0.07) comparing the changes of metabolites between healthy and post-S/RP periodontitis sites from gingival crevicular fluid (GCF) samples and periodontal soft tissue (solid tissue) samples.

DISCUSSION

In recent years, with the advent of the NMR and Mass Spectrometry (MS) technology, medical research has been focusing on the identification of metabolites as biomarkers for early detection and treatment of cancer.[20-26] The same way scientists have been starting to look at metabolite identification in saliva and GCF that can be used as biomarkers for diagnosis and treatment of periodontal diseases.[27,28] Saliva contents are convenient biomarkers for the diagnosis of periodontitis because inexpensive and fast to harvest. Several studies have looked at differences in metabolite levels revealed in saliva between healthy and periodontitis subjects.[29-35] A good tendency to separation with a variance ranging 20-40% between healthy and periodontitis groups was reported using PCA and PLS-DA models. These findings are matching our results for the gingival tissue metabolites, which showed a variance of 32.3% (Fig. 1) comparing sites with residual periodontitis after S/RP and healthy sites.

Saliva as diagnostic fluid for periodontal diseases has several advantages including low collection difficulty and non-invasiveness in sampling. However, saliva sample lacks specificity to periodontitis and it can be only used at a patient-level.[10] On the other hand, even if minimally invasive and technique sensitive, GCF may be a more attracting diagnostic tool for scientists because it comes specifically from local periodontal sites providing information at a site-level. The metabolite contents in GCF have also been considered potential indicators for periodontal status, demonstrating to be capable of distinguishing between healthy and periodontitis subjects.[36-38] However, the method of using NMR for the identification of metabolites in the GCF is still missing in the literature, which makes the comparison of our results limited.

Increased level of specific metabolites identified through MS in the GCF in deeper PDs were found, with a PCA confirming a clear distinction between healthy sites and deep PDs.[9] This is in line with our results on the gingival tissue metabolites revealed through NMR, showing both the PCA and PLS-DA with a separation between groups of healthy and post-S/RP periodontitis sites (Fig. 1).

In this pilot study, the gingival tissue metabolites were also compared to GCF metabolites of matched healthy and post-S/RP periodontitis sites from subjects recruited from a parallel ongoing longitudinal study (Fig. 3). A positive correlation, approaching statistical significance ($p=0.07$), was found comparing the levels of metabolites between healthy and post-S/RP periodontitis sites from GCF and gingival tissue samples (Fig. 4). These results are suggestive that the changes of metabolites in post-S/RP periodontitis sites compared to healthy ones may have similar pattern between GCF and gingival tissue.

CONCLUSIONS

Results from this pilot study are suggestive that 1) the changes of metabolites in the GCF are positively correlated with the ones in the gingival tissue when comparing healthy and post-SRP periodontitis sites and 2) the metabolites present in the periodontal soft tissue can discriminate between healthy and post-S/RP periodontitis sites. Future investigations should aim to obtain a greater sample size which will certainly increase the power of this pilot study.

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EXTENDED DISCUSSION

The periodontal probe is still considered the instrument of choice in assessing periodontitis, but the ability of the probe to accurately measure the depth of the pockets has been questioned.[9–12] The clinical characteristics of periodontitis measurable are CAL loss, RBL, PDs and BoP.[1,2] Since current methods of evaluation of periodontal disease aim at assessing a past history of disease, the periodontal research is increasingly focusing on identifying genetic, microbial, and host response-associated markers that distinguish between periodontitis phenotypes, or which can detect the onset and progression of periodontal diseases. In recent years, with the advent of the NMR and MS technology, scientists have been looking at metabolite identification in saliva and GCF that can be used as biomarkers for diagnosis, treatment and monitoring of the disease.

Saliva contents may be considered convenient biomarkers for the diagnosis of periodontal diseases because inexpensive and fast to harvest. Several studies have looked at differences in metabolite levels revealed in saliva between healthy and periodontitis subjects.[61-64] A good tendency to separation with a variance ranging 20-40% between healthy and periodontitis groups was reported using PCA and PLS-DA models. These findings are matching our results for the gingival tissue metabolites, which showed a variance of 32.3% (Fig. 1a and 1b) comparing periodontitis and healthy sites. In addition, regardless the changes in the salivary metabolite levels before and after initial periodontal non-surgical therapy, a distinctive pattern of metabolites has been demonstrated in the saliva of periodontitis subjects in comparison to non-periodontitis ones.[65] This important data strengthen our results comparing healthy and post-S/RP periodontitis subjects to identifying metabolites that

can discriminate between healthy and diseased status. A recent systematic review reported that valine, phenylalanine, isoleucine, tyrosine and butyrate have higher levels in saliva of periodontitis subjects compared to healthy ones.[66] Cadaverine, 5-oxoproline and histidine have also been identified in saliva and associated with periodontitis sites, potentially being predictors of the onset of periodontal disease.[67] These data are very encouraging for the use of oral fluids such as saliva as indicators for early detection and treatment of periodontal diseases.[68]

Nonetheless, although saliva as diagnostic fluid for periodontal diseases has several advantages including low collection difficulty and non-invasiveness in sampling, there is lack of specificity to periodontitis and it can be only used at a patient-level.[28] On the other hand, even if minimally invasive and technique sensitive, GCF may be a more attracting diagnostic tool for scientists because it comes specifically from local periodontal sites providing information at a site-level. The metabolite contents in GCF have also been considered potential indicators for the periodontal status, demonstrating to be capable of distinguishing between healthy and periodontitis subjects.[69] Different methods for analysis have been used for GCF, including MS (coupled with liquid or gas chromatography), enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay and others.[70] However, the method of using NMR for the identification of metabolites in the GCF is still missing in the literature, which makes the comparison of our results not possible.

The most prevalent periodontitis-specific biomarkers revealed in the GCF were highly reactive compounds such as malondialdehyde and 8-hydroxy-deoxyguanosine, according to a systematic review with meta-analysis.[70] The purine degradation pathway, an integral part of the reactive oxygen species (ROS) production, has been found significantly accelerated in the GCF of periodontitis sites.[55] These findings

confirmed the association between periodontal destruction and products derived from oxidative stress.[71,72] It has been widely reported in the literature that ROS, produced by polymorphonuclear leukocyte (PMN) cells during the inflammatory response, are involved in the pathogenesis of periodontal breakdown.[73] Differences in the metabolites present in the GCF revealed through chromatography-MS between generalized aggressive periodontitis (GAgP) and healthy sites was also demonstrated.[56] Noradrenaline, Uridine, α-tocopherol, dehydroascorbic acid, xanthine, galactose, glucose 1-phosphate and ribulose 5-phosphate were all associated with GAgP sites. Another study identified through MS increased level of specific metabolites in the GCF in deeper PDs, with a PCA confirming a clear delineation between healthy sites and deep PDs.[46] This is in line with our results on the gingival tissue metabolites revealed through NMR, showing both the PCA and PLS-DA with a separation between groups of healthy and periodontitis sites (Fig. 1).

The present investigation aimed to access cross-sectionally if specific metabolites revealed through NMR in gingival tissues were diagnostic of periodontitis. Twenty-seven metabolites were identified in gingival tissue biopsies of 7 healthy and 9 post-S/RP periodontitis subjects. Aspartate showed a trend in projection for statistical significance and it has been found in higher levels in post-S/RP periodontitis sites compared to healthy ones (Fig. 3). Considering the limitation in the sample size which may have affected the statistical significance, this result may be promising. In addition, to the author's knowledge, there are no other studies that have been carried out cross-sectionally or longitudinally evaluating the metabolites present in the gingival tissues of healthy and periodontitis subjects. Therefore, direct comparisons to the results from the literature cannot be performed.

In this pilot study, the gingival tissue metabolites were also compared to GCF metabolites of matched healthy and post-S/RP periodontitis subjects recruited from a parallel ongoing longitudinal study (Fig. 3). A positive correlation between the changes of metabolites in the GCF and the ones in the gingival tissue was found when comparing healthy and post-S/RP periodontitis sites (Fig. 4), with a p-value approaching statistical significance ($p=0.07$). These results are suggestive that the changes of metabolites in post-S/RP periodontitis sites compared to healthy ones may have similar pattern between GCF and gingival tissue. This may justify the research in the GCF to identifying the metabolites that discriminate between healthy and periodontitis sites, which is certainly a less invasive method compared to harvesting gingival tissue biopsies. However, the limited sample size and the impossibility to compare these findings with the literature prevent us to draw definitive conclusions. With an increased utilization of the NMR already occurring in the cancer research, it is the author's expectation that data will be available soon also in the periodontal research.[74] In the medical field, the metabolite profiling through NMR has already led to discovering important biomarkers for early detection and prognosis of thyroid cancer and long-term risk of breast cancer.[75,76] Other studies using NMR technology are still ongoing aiming for the detection of potential biomarkers that may contribute for early diagnosis and treatment for diseases such as pancreatic cancer, prostate cancer, colorectal cancer, haematologic malignancies among others.[77-80] Future application of NMR for the analysis of both GCF and gingival tissue should aim to obtain more information regarding potential biomarkers for an early detection and characterization of periodontal diseases, with a possible prognostic and predictive value.

CONCLUSIONS

Results from this pilot study are suggestive that 1) the changes of metabolites in the GCF are positively correlated with the ones in the gingival tissue when comparing healthy and post-SRP periodontitis sites and 2) the metabolites present in the periodontal soft tissue can discriminate between healthy and post-S/RP periodontitis sites.

Nonetheless, in this study a direct comparison to establish whether the levels of metabolites in the periodontal tissues were similar to the ones present in GCF comparing healthy versus post-S/RP sites was not possible. The limitation of this pilot study resides in comparing the data from different patients' sample, even though the periodontal sites (diseased versus healthy) were carefully matched.

Future investigations should aim to directly compare the periodontal tissue metabolites with the GCF ones obtained from the same patients' sample, which will certainly increase the power of the study.

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