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Salivary metabolomic profile in adolescents with juvenile systemic lupus erythematosus

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Abstract: The aim of this study was to characterize the salivary metabolomic profile in adolescents with juvenile systemic lupus erythematosus (jSLE). A total of 24 adolescents with jSLE (15.92 ± 2.06 years) and 12 systemically healthy controls (15.25 ± 2.7 years) were included in the study. Participants underwent rheumatologic testing and periodontal examination, with the recording of plaque index (PI), probing depth (PD), clinical attachment level (CAL), and bleeding on probing index (BPI). Unstimulated whole saliva was collected from both groups and stored at -80°C . The salivary proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectra were acquired in a spectrometer operating at 500 MHz. Partial least squared discriminant analysis (PLS-DA) and orthogonal PLS-DA (O-PLS-DA) were used for statistical analysis. Mean CAL and PI were significantly increased in the group with jSLE ($p < 0.01$). Patients with jSLE presented a significantly different salivary metabolic profile (accuracy = 0.54; $R^2 = 0.86$; $Q^2 = -0.293$), significantly higher salivary levels of N-acetyl sugars, and significantly reduced levels of phenylalanine, glycine, taurine, hydroxybutyrate, and valerate compared with healthy controls ($p < 0.05$). It is suggested that the salivary metabolomic profile analyzed by $^1\text{H NMR}$ in patients with jSLE presents a different fingerprint that the systemically healthy subjects. Integrating the variation of metabolites with the identification of the metabolic pathways involved seems to provide a better understanding of the influence of systemic disease on salivary metabolites.

Keywords: Gingivitis; Metabolomics; Saliva; Adolescent; Magnetic Resonance Spectroscopy.

Introduction

Juvenile systemic lupus erythematosus (jSLE) is an autoimmune disease that can cause significant damage, disability, or death. Its onset occurs before the age of 18 years, it affects approximately 20% of patients with adult-onset systemic lupus erythematosus (SLE), and it can be more aggressive than SLE, with higher disease activity and medication burden.^{1,2} The pathogenic mechanisms underlying SLE have not been completely elucidated,³ but it is believed that SLE results from a complex interaction between genetic, epigenetic, hormonal, and environmental



factors, leading to the activation and dysregulation of the immune response, which promotes the formation of autoantibodies.⁴

Patients with SLE have a higher prevalence of periodontal disease⁵ compared with systemically healthy patients. The factors that correlate with SLE and periodontal diseases have not been fully elucidated. Due to common pathogenic pathways, it is possible that some key metabolites are associated with both diseases. Despite having different etiologies, SLE and periodontal diseases share common pathogenic characteristics,⁶ such as hyperactivity of B lymphocytes, high production of IgG antibodies, genetic, environmental and immunological influences,⁷ production of oxygen free radicals (OFR),⁸ increased activity of matrix metalloproteinases (MMPs),⁹ high levels of anti-neutrophil cytoplasmic antibody (ANCA), and altered cytokine and tumor necrosis factor-alpha (TNF- α) production.⁵ Therefore, understanding the metabolic pathway associated with these diseases is important to understand the clinical phenotypes.

Recently, Robinson et al.¹⁰ performed a multi-omics analysis in patients with jSLE and found that a high ApoB:ApoA1 is a potential biomarker of increased cardiometabolic risk and worse clinical outcomes, which, according to the authors, could help identify patients that require increased disease monitoring or lifestyle changes.¹⁰ Therefore, metabolomics could be a potential diagnostic tool and treatment guide for patients with jSLE.

Metabolomics deals with omics technologies that identify and quantify metabolites from cells, tissues, body fluids, or systems.^{11,12} The metabolomics profile reflects the low molecular weight metabolic phenotype, that is, the metabolites produced as a dynamic response to genetic background as well as physiological, pathological, developmental and environmental stimuli in a living system.¹³ In a recent literature review, Zhang and Mohan show how metabolomics studies have shed substantial light on the pathophysiology of SLE. They reported that metabolomics studies of SLE should also consider potential contributions of confounders, such as medications, co-morbidities, smoking, and diet,¹⁴ re-enforcing the need for more studies

investigating the metabolomic profile in such patients and that confounders should be taken into consideration.

Gardner et al.¹⁵ suggest that researchers working on metabolomics should be encouraged to consider the use of saliva, as the collection, handling, and preparation for metabolomics analysis has been fairly well researched and is relatively simple. Therefore, the aim of this study was to analyze the profile of salivary metabolites in adolescents with jSLE and gingivitis and compare them with systemically healthy adolescents with gingivitis by proton nuclear magnetic resonance (¹H-NMR).

Methodology

The study protocol was approved by the Ethics Committee of Pedro Ernesto University Hospital (UERJ, Rio de Janeiro, Brazil: 380.686/2013 and amendment 2.284.225/2017). The legal guardians or the patients themselves, if they were of legal age, signed the informed consent form before participating in the study.

Study population and sample collection

The participants were recruited at the participants were recruited at the Center for Adolescent Health Studies (Núcleo de Estudos da Saúde do Adolescente - NESA), UERJ, Rio de Janeiro, Brazil. To participate in the test group, individuals had to be between 12 and 18 years old and been diagnosed and under treatment/follow-up for jSLE, according to the Systemic Lupus International Collaborating Clinics (SLICC) criteria¹⁶ with the team of rheumatologists at the Center for Adolescent Health Studies.¹⁷ For the control group, individuals receiving treatment at NESA but did not have any systemic disease were also selected. All eligible individuals from this center were recruited for this study. The NESA is a reference center for SLE where individuals diagnosed with jSLE and SLE routinely return for follow-up medical appointments, with a mean treatment/follow-up period of 2 years. The patient with the longest follow-up had 5 years. Systemically healthy individuals in a similar age range constituted the control group. The final sample consisted of 12 individuals with jSLE (11 women) and

12 controls without systemic disease (12 women). Afterwards, they were referred for evaluation of the oral condition at the Dentistry Clinic of the State University of Rio de Janeiro (UERJ). The recruitment period was 1 year.

For each participant, a medical record was filled out containing personal data, anamnesis, general health, rheumatological parameters, and oral data. The rheumatological data of patients with *JSLE* were filled out by a rheumatologist from the Center for Adolescent Health Studies (F. R. Sztajnbok), who was responsible for the treatment and monitoring of these individuals. The dental exams performed were Decayed, Missing, and Filled Teeth (DMFT) index, plaque index (PI), probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP) on all teeth, except third molars.

All individuals were required to have at least 20 spontaneously erupted teeth (excluding third molars) and have been diagnosed with gingivitis according to the 2018 classification of periodontal and peri-implant diseases and conditions.¹⁸ Exclusion criteria were pregnancy, lactation, current orthodontic treatment, or periodontal treatment in the last 6 months. In the control group, patients with any systemic disorder that could affect the periodontal support apparatus¹⁹ were also excluded.

To perform the periogram (PI, PD, CAL, and BOP), each tooth was evaluated under artificial lighting, with relative isolation, and air drying. A millimeter periodontal probe (North Carolina Universal 15 Single Point - PCPUNC15 - Hu -Friedy®, Chicago, USA) was gently inserted into the gingival sulcus/periodontal pocket to obtain PD, CAL, and BOP measurements. With the probe at its final position (resistance point at the bottom of the sulcus/pocket), PD and CAL measurements were recorded. The PD measurement was taken from the distance between the gingival margin and the bottom of the sulcus, and the CAL was given by the distance between the bottom of the pocket and the cement-enamel junction (CEJ). The BOP used in this study was dichotomous (presence/absence), obtained from probing four sites/teeth (mesiobuccal, buccal, distobuccal, and palatine), and the BOP index was

calculated by obtaining the percentage of bleeding sites from the total of sites probed.

Sample collection

Saliva was collected before the dental exams, so samples were not contaminated by blood from gingival tissues during the exams. Patients were instructed to not eat or drink for at least 30 minutes before saliva collection. Then, unstimulated whole saliva collection was performed by expectorating from the mouth directly into a sterile plastic collection tube a volume of 4 mL of saliva. The saliva samples of all the subjects were taken simultaneously (8:00 am to 10:00 am) to avoid fluctuation in the results due to the circadian saliva cycle. For each patient, 1 mL of saliva was transferred to a pressure Eppendorf™ tube containing 20 µL of protease inhibitor (SIGMAFAST™ Protease Inhibitor Tablets, Sigma Aldrich Co., St. Louis, USA) to be centrifuged (Micro-NT800 centrifuge, Novatecnica®) for 8 minutes at 8000 rotations per minute at laboratory temperature (25°C). After centrifugation, the saliva supernatant was transferred to a new Eppendorf tube and frozen at -80°C until the samples were processed.

Sample preparation and NMR measurements

After storage at -80°C, the collected samples were unfrozen at 25 °C. Then 500 µL of each salivary sample was mixed with 100 µL of sodium phosphate buffer (pH 7.0) containing deuterated water at a concentration of 10% (D₂O; Cambridge Isotope Laboratories Inc., Tewksbury, USA) and 5 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS; Sigma-Aldrich, Milwaukee, USA). The D₂O was used for the recognition of the magnetic field in the sample, and the DSS was used for the internal reference of chemical displacement ($\delta = 0.00$ ppm).^{11,12,20}

The spectra were obtained according to our previous studies^{11,12,20-22} and a 500 MHz Avance III NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) at 25°C was used at the Jiri Jonas National Nuclear Magnetic Resonance Center (CNRMN). The CPMG pulse sequence (Carr-Purcell-Meibom-Gill) was used for the ¹H spectrum acquisition, with water suppression. Gradients with 1024 scans were used. The total correlation experiments ¹H-¹H-TOCSY

were used to confirm the reports and resolve the ambiguous metabolites, using 2048 X 256 points, spectral width of 12,019 Hz in each dimension and a mixing time of 70 ms.

After spectra acquisition, the edge effects were evaluated by overlaying all spectra using Topspin (Bruker Biospin, Rheinstetten, Germany). The spectra and spectra regions that could not be corrected for phase and baseline were excluded from the analysis.

Statistical analyses

Each NMR spectrum was analyzed, integrating the 0.03 ppm size regions. The water region was excluded from the analyses (4.50 to 5.15 ppm), as were the spectral regions that could not be corrected for the phase and baseline (2.69 to 2.73 ppm). Resonance assignments were made based on our previous studies,^{11,12,20-22} the Human Metabolome Database (<http://www.hmdb.ca/>), and confirmed using the assignments that were made from the ¹H-¹H-TOCSY experiments. The data set was stored in a matrix with the saliva samples occupying the lines and the chemical shifts of the metabolites arranged in columns (284 shifting regions). Metabolic data were extracted using the AMIX Statistics statistical program (version 3.9.14, Bruker Biospin, Rheinstetten, Germany). The spectra and data extraction are available on Open Science Framework (<https://osf.io/DOI.10.17605/OSF.IO/CHK2V>).

The data were normalized by summing the peak intensities and using Pareto. The multivariate analyses to determine the most relevant metabolites for groups with and without SLE were performed in Metaboanalyst (version 4.0) with partial least squared discriminant analysis (PLS-DA) and orthogonal PLS-DA (O-PLS-DA), which is a supervised classification method that uses the group label to maximize the separation between different groups. The VIP score was analyzed to define the different metabolites in the groups. The t-test was applied to perform univariate statistics and verify whether there was a difference in metabolites between patients with and without SLE. To define the performance of the model, the parameters Q² (fraction of variation Y predicted by model X), R² (fraction of variation

X modelled), and accuracy (ACC) for each set of projections were used. The 95% confidence interval was used for the models.

The clinical and demographic data were analyzed using Statistical Package for Social Sciences (SPSS) version 23.0 (SPSS Inc., Chicago, USA) by applying the Mann-Whitney and chi-square tests. Statistical significance was assessed using the t-test for comparisons between groups. A value of $p < 0.05$ was considered a statistically significant difference.

Results

Both groups had 12 individuals; demographic data, general health and rheumatologic parameters, and oral data are shown in Table 1. The age and gender of the patients in the two groups were similar. The mean time of jSLE diagnosis time was 2.25 (\pm 1.65) years. Total leukocytes, eosinophils, and glycemia differed between jSLE and control groups ($p < 0.05$). There were no smokers in any of the groups. Items related to oral health habits reported by the patients (frequency of brushing and flossing) and their perception of halitosis showed no distinction between the groups.

Table 2 presents the periodontal condition data. The variables statistically different between the groups with and without jSLE were mean CAL and PI = 0. The jSLE group had higher PI compared to the control. Despite the difference between the groups, the CAL did not reflect losses associated with periodontitis. Bone resorptions were predominantly on the buccal and lingual/palatal surfaces, measuring up to 3 mm, not associated with PD greater than 3 mm (data not shown), thus not configuring periodontitis. The BOP was similar between the groups and did not present statistical significance.

Figure 1 shows the main metabolites (according to the PLS-DA VIP score) and the differences between groups. In the jSLE group there was reduced levels of phenylalanine, valerate, glycine, taurine, and hydroxybutyrate and increased levels of N-acetyl sugars, and a metabolite that presented ambiguous results in the jSLE group. The graph from the PLS-DA results (Figure 1A), shows that two components presented a slight difference between groups. The same analysis using components

Table 1. Demographic, general health and rheumatologic parameters, medicine consumption, and oral characteristics of individuals with juvenile systemic lupus erythematosus (jSLE) and the control group without jSLE.

Characteristic	jSLE (n = 12)	Control group (n = 12)	p-value*
Demographic data - mean (\pm standard deviation)			
Age	15.92 (\pm 2.06)	15.25 (\pm 2.7)	0.59
Female	11 (91.66%)	12 (100%)	1.00
Male	1 (8.33%)	0 (0%)	
Smokers	0	0	1.00
General health and rheumatologic parameters - mean (\pm standard deviation)			
Age of diagnosis	13.66 (\pm 0.98)	NA	NA
Diagnosis time	2.25 (\pm 1.65)	NA	NA
SLEDAI	4.00 (\pm 6.15)	NA	NA
SLICC	1.58 (\pm 4.56)	NA	NA
Erythrocytes	4.57 (\pm 0.40)	4.58 (\pm 0.35)	0.93
Hemoglobin g/dL	12.55 (\pm 0.74)	12.36 (\pm 1.25)	0.67
Hematocrit %	35.03 (\pm 10.32)	37.40 (\pm 3.19)	0.46
Mean corpuscular volume (MCV)	84.13 (\pm 4.64)	76.773 (\pm 17.12)	0.19
Mean corpuscular Hemoglobin (MCH)	32.57 (\pm 16.93)	33.53 (\pm 15.69)	0.89
Total leukocytes	5,450 (\pm 2,949.20)	8,363.63 (\pm 2,459.78)	0.02
Basophils	1.27 (\pm 3.13)	0	0.20
Eosinophils	1% (\pm 1.18%)	3.55% (\pm 5.05%)	0.13
Platelets	307,500 (\pm 82,383.25)	286,218.18 (\pm 28,435.25)	0.40
Glycemia	82.27 (\pm 6.35)	91.8 (\pm 10.45)	0.03
Medicine consumption – Number of individuals (% percentage)			
	12 (100%) of which:	10 (83.3%) of which:	0.17
	8 (66.67%) analgesic	6 (50.00%) analgesic	0.57
	1 (8.33%) NSAIDs	0 (0.00%) NSAIDs	NA
Use of medications	4 (33.33%) Omeprazole	3 (25%) Omeprazole	0.76
	3 (25.00) ATB	1 (8.33) ATB	0.33
	7 (58.33%) Immunosuppressants drugs	0 (0.00%) Immunosuppressants	NA
	7 (58.33%) corticosteroids	0 (0.00%) corticosteroids	NA
	9 (75.00%) antimalarial agent	0 (0.00%) antimalarial agent	NA
Oral characteristics – Number of individuals (% percentage)			
	2 (16.7%) - mouth	0 (0%) - mouth	
Breath	7 (58.3%) - nasal	5 (41.7%) - nasal	0.14
	3 (25%) - both	7 (58.3%) - both	
	4 (33.3%) - 2x/day	6 (50%) - 2x/day	
Brushing frequency	8 (66.7%) - 3x/day or more	6 (50%) - 3x/day or more	0.68
Flossing	3 (8.3%)	6 (50%)	0.07
Halitosis	3 (25%)	4 (33.3%)	0.59

NSAIDs: non-steroidal anti-inflammatory drugs; ATB: antibiotics; NA: Not applicable. Categorical data was analyzed with Chi-square test and quantitative data with Mann-Whitney test. The level of statistical significance considered was 5% ($p \leq 0.05$); Statistically significant data are highlighted in bold.

Table 2. Dental and periodontal characteristics of individuals with jSLE and the control group without jSLE.

Characteristics	jSLE (n = 12)	Control group (n = 12)	p-value
Mean of teeth	27.67 (± 1.15)	27.67 (± 0.89)	0.80
PD (mm)	2.21 (± 0.33)	2.16 (± 0.28)	0.35
% PD ≤ 3mm	98.36 (± 2.65)	97.95 (± 2.78)	0.20
% PD 4–5mm	1.64 (± 2.65)	2.05 (± 2.78)	0.20
CAL (mm)	0.08 (± 0.09)	0	< 0.01
PI	47.32	17.86	< 0.01
BOP	14.60 (± 10.09)	13.26 (± 5.54)	1.00

PD: probing depth; CAL: clinical attachment level; PI: plaque index; BOP: bleeding on probing. The level of statistical significance considered was 5% ($p \leq 0.05$); data are represented as means and standard deviation; the PI is represented by the median and its respective standard deviation.

Table 3. Statistically significant metabolites according to univariate and multivariate analyses.

Metabolite HMDB card	δ H (ppm)	jSLE group (n=12)			Control group (n=12)			p-value
		Median	Minimum	Maximum	Median	Minimum	Maximum	
Phenylalanine ^{AB} (Phe I) HMDB0000159	7.39	09.53 x 10 ⁻⁴	01.60 x 10 ⁻⁴	14.72 x 10 ⁻⁴	13.74 x 10 ⁻⁴	0.88 x 10 ⁻⁴	19.37 x 10 ⁻⁴	0.02
Phenylalanine ^{AB} (Phe II) HMDB0000159	7.36	19.02 x 10 ⁻⁴	12.70 x 10 ⁻⁴	30.67 x 10 ⁻⁴	30.57 x 10 ⁻⁴	04.67 x 10 ⁻⁴	47.91 x 10 ⁻⁴	0.02
Valerate ^{AB} HMDB0000892	0.88	81.11 x 10 ⁻⁴	50.35 x 10 ⁻⁴	115.88 x 10 ⁻⁴	104.15 x 10 ⁻⁴		215.17 x 10 ⁻⁴	0.03
Glycine ^{AB} HMDB0000123	3.55	90.75 x 10 ⁻⁴	65.57 x 10 ⁻⁴	13.01 x 10 ⁻⁴	113.67 x 10 ⁻⁴	28.08 x 10 ⁻⁴	229.35 x 10 ⁻⁴	0.05
Taurine ^{AB} HMDB0000251	3.43	29.42 x 10 ⁻⁴	04.90 x 10 ⁻⁴	56.88 x 10 ⁻⁴	35.77 x 10 ⁻⁴	17.77 x 10 ⁻⁴	88.55 x 10 ⁻⁴	0.06
Phenylalanine ^A (Phe III) HMDB0000159	7.42	09.29 x 10 ⁻⁴	0.10 x 10 ⁻⁴	12.77 x 10 ⁻⁴	11.91 x 10 ⁻⁴	0.73 x 10 ⁻⁴	19.29 x 10 ⁻⁴	0.06
N-acetyl sugars ^A HMDB0000215	2.05	282.21 x 10 ⁻⁴	111.34 x 10 ⁻⁴	555.37 x 10 ⁻⁴	242.52 x 10 ⁻⁴	124.28 x 10 ⁻⁴	334.21 x 10 ⁻⁴	0.07
Hydroxybutyrate ^A (Hydroxybut II) HMDB0000011	2.47	0.0 x 10 ⁻⁴	0.0 x 10 ⁻⁴	08.58 x 10 ⁻⁴	2.98 x 10 ⁻⁴	0.0 x 10 ⁻⁴	17.92 x 10 ⁻⁴	0.08
Hydroxybutyrate ^A (Hydroxybut I) HMDB0000011	1.21	0.0 x 10 ⁻⁴	0.0 x 10 ⁻⁴	47.84 x 10 ⁻⁴	43.89 x 10 ⁻⁴	02.61 x 10 ⁻⁴	69.78 x 10 ⁻⁴	0.07
Ambiguous ^A	1.36	18.35 x 10 ⁻⁴	07.77 x 10 ⁻⁴	27.50 x 10 ⁻⁴	14.16 x 10 ⁻⁴	5.37 x 10 ⁻⁴	21.37 x 10 ⁻⁴	0.08

δ : chemical shift; A: significant metabolites ($p \leq 0.05$) in the multivariate analysis (VIP score of the PLS-DA analysis); B: significant metabolites in the univariate analysis. VIP score of the PLS-DA analysis was carried out between the test and control groups, their respective chemical shifts and the significance value obtained in the univariate analysis.

showed greater distinction between the groups, presenting an accuracy of 0.54, with R^2 of 0.86 and Q^2 of -0.293.

O-PLS-DA analysis (Figure 1B), in turn, showed a more expressive difference between groups. The salivary metabolic profile of the patients in the test

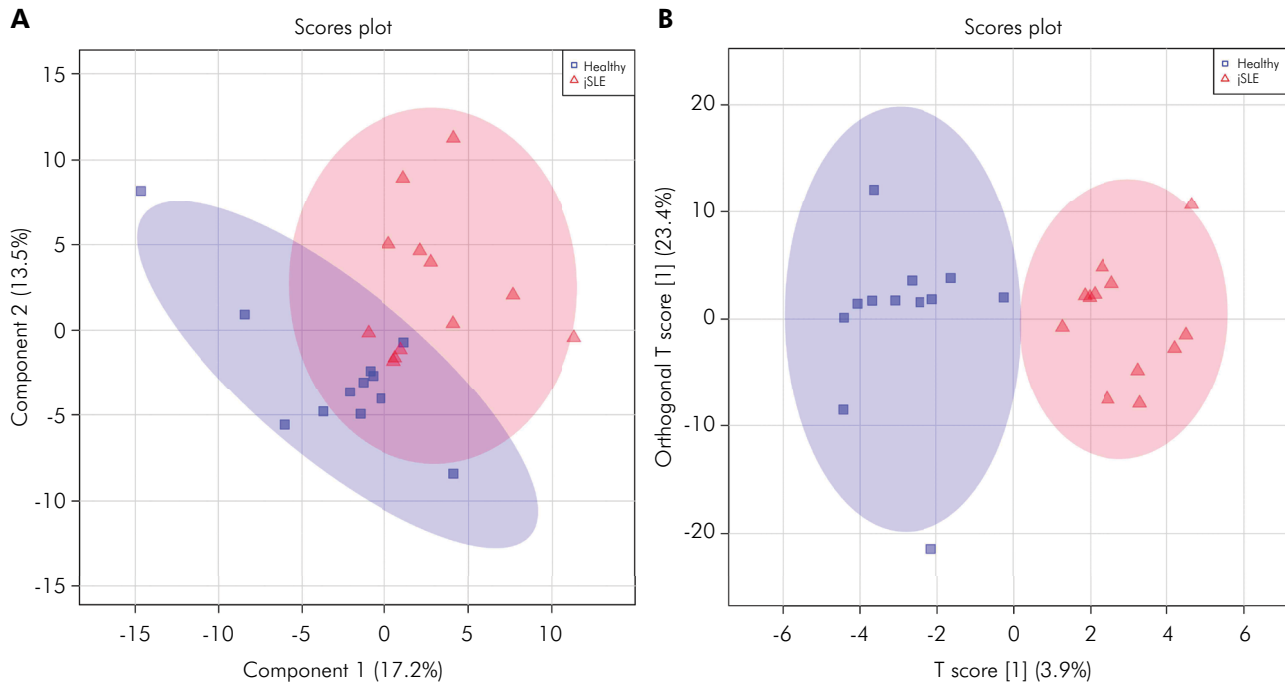


Figure 1. Graphical representations of the multivariate PLS-DA and O-PLS-DA.

group (with jSLE and gingivitis) was different from that of the patients in the control group (systemically healthy with gingivitis).

The data set stored in the matrix containing the chemical shifts (δH - shown in Table 3) of the metabolites observed in the PLS-DA VIP graph (Figure 2) were tracked. After identifying the chemical position of each metabolite, it was possible to perform the t-test and the descriptive analysis (median, minimum value, maximum value and significance) to compare the groups (Table 3). Not all metabolites identified in the PLS-DA VIP score showed statistical difference on the t-test. However, the descriptive analysis of the metabolites confirmed the differences in their concentration observed previously in Figure 2.

Phenylalanine showed three peaks of intensity in the VIP score, and all were higher in the control group than in the test group. The peak of phenylalanine ($\delta H = 7.39$ and $\delta H = 7.36$, respectively) with the highest intensity in the VIP score showed statistical difference ($p = 0.02$) in the univariate analysis, and the differences in concentration between the groups were confirmed in the descriptive analysis. Only phenylalanine, with the lowest intensity in the VIP score ($\delta H = 7.42$), was statistically insignificant in

the univariate analysis. Still, the descriptive analysis confirmed the differences in the concentration found in the VIP score.

The third highest peak in the VIP score was for valerate ($\delta H = 0.88$), whose statistical difference was significant ($p = 0.03$), and the differences in the descriptive analysis matched those found in the VIP score (highest in the control group and lowest in the test group). The same occurred with glycine ($\delta H = 3.55$ and $p = 0.05$).

Taurine ($\delta H = 3.43$) and hydroxybutyrate ($\delta H = 2.47$ and $\delta H = 1.21$) were different between groups both in the multivariate analysis of the VIP score and in the descriptive analysis (increased in the control group and reduced in the test group). It was non-significant in the univariate analysis.

The only metabolites with the highest concentrations in the test group and the lowest in the control group in the multivariate and descriptive analyses were N-acetyl sugars ($\delta H = 2.05$) and the metabolite with an ambiguous assignment ($\delta H = 1.36$). Neither, however, were significant in the univariate analysis.

Based on the main metabolites expressed in the multivariate analysis, an analysis of the metabolic pathways was performed (Figure 3). The pathways

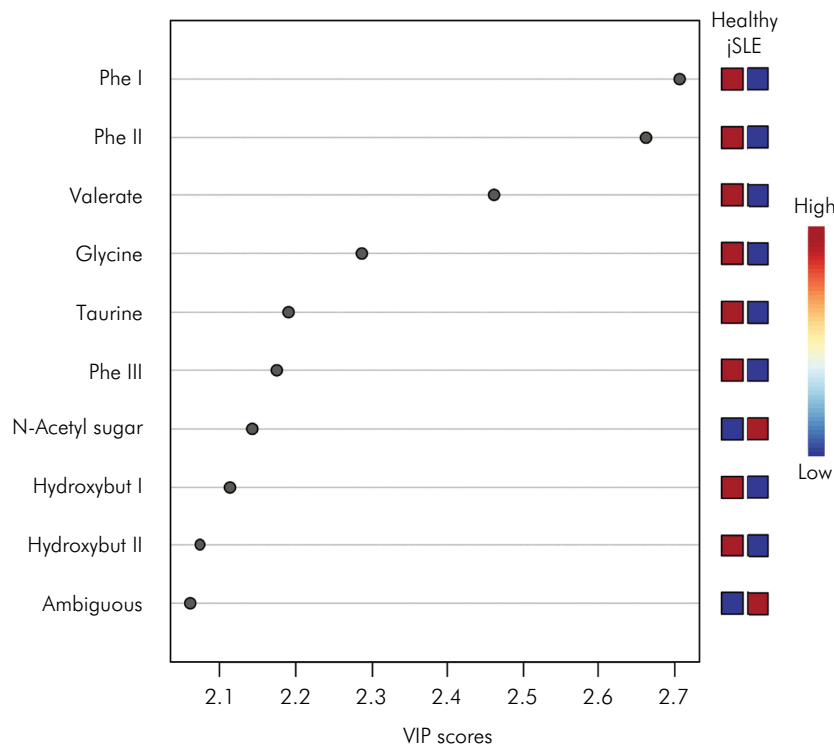


Figure 2. VIP score based on the PLS-DA model showing the metabolites that differed between groups and their displacements (x-axis); the heat map on the right indicates differences in metabolite concentration between groups.

involved in the metabolism and biosynthesis of amino acids were those that had the greatest impact on the metabolic changes observed in the test group (Figure 3: A = biosynthesis of phenylalanine, tyrosine and tryptophan; B = metabolism of taurine and hypotaurine; C = phenylalanine metabolism), while an intermediate impact can be attributed to the metabolism of glycine, serine, and threonine (Figure 3D). The identified low-impact pathways were those of glyoxylate and dicarboxylate metabolism (Figure 3E) and glutathione (Figure 3F), and the least impacted pathway was that of bile acids biosynthesis (Figure 3G).

Discussion

jSLE is a rare but severe autoimmune disease. Its onset occurs before the age of 18 and affects approximately 20% of SLE patients. jSLE is more aggressive than adult-onset SLE, with higher disease activity and medication burden. The only study about

metabolomics analysis in jSLE patients assessed cardiovascular disease risk and identified complex patterns of atherogenic dyslipidemia in JSLE patients associated with inflammation.²³ Due to the scarcity of studies on jSLE, the closest comparisons are with SLE. One study about metabolomic activity in SLE revealed that the disease influences energy, amino acid, lipid, and purine metabolism. The study demonstrated that SLE patients had decreased citrate, pyruvate, phosphocoline, and amino acid levels (alanine, tyrosine, isoleucine, valine, phenylalanine, lysine, histidine, glutamine). On the other hand, in accordance with the results of Robinson et al. (2021), N-acetyl glycoprotein, very-low density lipoprotein, and low-density lipoprotein were upregulated.²⁴ Our study demonstrated similar results in phenylalanine and N-acetyl sugars abundancies.

As far as we know, our study is the first to assess salivary metabolome in jSLE patients. This study demonstrated that the salivary metabolic profile of patients with jSLE was different from the profile

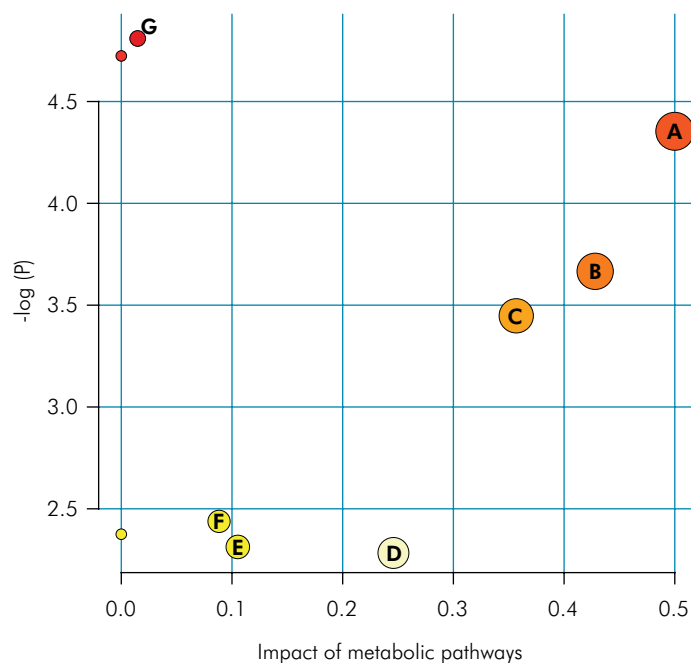


Figure 3. Metabolic pathways analyzed using the metabolites important to group distinctions (Table 2) prepared with Metaboanalyst. A = phenylalanine, tyrosine and tryptophan biosynthesis; B = metabolism of taurine and hypotaurine; C = phenylalanine metabolism; D = metabolism of glycine, serine and threonine; E = glyoxylate and dicarboxylate metabolism; F = glutathione metabolism; G = biosynthesis of bile acids.

of systemically healthy patients, using $^1\text{H-NMR}$. Seven metabolites were altered: phenylalanine, glycine, taurine, valerate, and hydroxybutyrate were decreased and N-acetyl sugars and ambiguous assignment ($\delta = 1.36$ ppm) were increased in the test group. The oral condition of patients with jSLE corroborates the literature that demonstrates that individuals with SLE have higher values of CAL and visible dental plaque.¹⁷

The analysis of the metabolic reactions connecting the immune-mediated inflammatory disease (IMID)-associated metabolites showed a relationship between the citric acid cycle, phenylalanine, and glycine-serine metabolism pathways.²⁵ Among the metabolites responsible for the distinction between the groups, phenylalanine was highlighted, and it was reduced in patients with jSLE compared to systemically healthy individuals. These results suggest that these changes in the phenylalanine levels were related to jSLE. Our findings are in line with metabolomic studies in SLE.²⁶ It has been suggested that the reduction of serum levels of phenylalanine in individuals with SLE is associated with amino acid

degradation processes directed to the regulation of biological functions, including inflammatory and autoimmune responses.²⁶ Zhang et al.¹⁴ reported an association between the presence of phenylalanine and the use of glucocorticoids (GCs) and multiple immunosuppressants by patients with SLE.

Our data suggest that phenylalanine reduction is expressive in subjects with jSLE, since it was observed even in the presence of local diseases, such as gingivitis. The literature reports an increase in phenylalanine or hydrocinnamate, an analog of phenylalanine, in systemically healthy patients with periodontal disease.^{27,28} Aimetti et al.²⁷ and Romano et al.²⁹ found increased levels of phenylalanine in the saliva of patients with generalized chronic periodontitis, which was associated with the degradation of the host's tissues. Ozeki et al.³⁰ found increased levels of phenylalanine in the gingival fluid of patients with periodontitis whose diseased sites had deep pockets compared to those with moderate pockets of healthy sites, using analysis by gas chromatography - mass spectrometry (GC-MS). Romano et al.²⁹ also found increased levels of phenylalanine in the saliva of

patients with generalized aggressive periodontitis. Increased levels of phenylalanine in the gingival fluid of sites with periodontal disease have been associated with interactions between host tissues and biofilm bacteria.³¹ Altered levels of phenylalanine and tyrosine and their byproducts were found in the saliva of patients and were associated with pocket depth.³² In our study, the subjects presented with gingivitis, therefore without periodontal pockets. The present study demonstrated reduced levels of phenylalanine in patients with jSLE compared to systemically healthy individuals, corroborating previous data. However, since both groups with and without jSLE had gingivitis, it was not possible to estimate the influence of gingivitis on this finding. The lack of a group without gingivitis can be considered a limitation of the study. However, patients with SLE have a high prevalence of gingivitis.²⁶ For this reason, to check the metabolites associated with systemic disease, a control group with the same conditions (with gingivitis but systemically healthy) was selected. SLE has a high systemic impact on salivary metabolites, since in our study, both groups presented gingivitis and phenylalanine was decreased in patients with jSLE. We suggest that the systemic condition impacted salivary metabolomics more than the local disease (*i.e.*, gingivitis).

We found significantly lower glycine levels in patients with jSLE than healthy controls. Glycine is an amino acid found in collagen that provides flexibility for its active sites, and is related to a variety of important metabolites, such as glutathione, creatine, purines, serine, and hemoglobin heme.³³ Also, using GC/MS, the literature shows reduced levels of amino acids, including glycine, in the serum of patients with SLE compared with healthy individuals, which is in line with our findings.³⁴ Alonso et al.²⁵ used NMR to analyze the urinary metabolome of a large group of patients with six immune-mediated inflammatory diseases, including SLE, comparing them with healthy controls, and found that the glycine-serine pathways were hyperactivated. From these results, it was suggested that glycine could be a metabolite highly informative of the inflammatory processes that characterize immune-mediated inflammatory

diseases. These contradictory findings could be explained by studies on biofluid, since urine is the result of physiologic excretion.

Taurine levels were lower in the jSLE group. Taurine performs important functions, such as the stabilization of cell membrane structures,³⁵ bile salt conjugations, antioxidation,³⁶ immunomodulation, and inflammatory mediators regulation.³⁷ It was found at lower levels in the urine of patients with SLE and class III and IV lupus nephritis³⁸ compared to those with class V. Reduced levels of taurine have been associated with attempts by these patients to manage or repair the existing kidney pathology, since the reserve of taurine in the body is regulated by the kidneys. Moreover, taurine acts as an antioxidant and is even used to treat kidney disorders. Barnes et al. found the metabolic pathways of amino acids and dipeptides hyper-regulated in their study.²⁸ Our study demonstrated that the second most important pathway was the metabolism of taurine and hypotaurine. Taurine was one of the metabolites with the greatest salivary difference between the altered amino acids in the group with periodontitis (reduced) compared to the periodontally healthy control group, which is associated with the degradation of macromolecules.³⁰ It is suggested that taurine levels can also be altered in gingivitis; however, in this initial inflammatory process, this metabolite is present in reduced levels.

Hydroxybutyrate was also lower in the jSLE group than in healthy controls. In combination with other biochemical and clinical parameters, this metabolite may be a useful clinical indicator of subclinical glucose metabolism, since it is a ketonic body.³⁹ Wu et al.⁴⁰ compared the sera of individuals with SLE and healthy individuals using Liquid Chromatography - Mass Spectrometry (LC-MS) and CG-MS, and they observed that the energy derived from lipids was reduced in SLE, based on the significantly reduced levels of the B-oxidation intermediates, 1,2 propanediol, and 3-hydroxybutyrate. A study comparing patients with chronic and aggressive periodontitis found increased levels of 3-D-hydroxybutyrate in the control groups.⁴¹ Eventually, with the development of periodontal disease, hydroxybutyrate may be detected from bacterial metabolic activity.^{12,21}

The threonine and glutathione pathways had medium and low impact, respectively, in the results of this study. Barnes et al. associated reduced levels of glutathione with oxidative stress when they analyzed the gingival fluid of patients with different periodontal conditions.³¹ N-acetyl sugars were found to be increased in the jSLE group in our study. Rzeznik et al.⁴¹ found increased N-acetylated groups in the saliva of patients with chronic and aggressive periodontitis compared to healthy controls. The literature shows reduced levels of N-acetyl groups in the saliva of patients with periodontitis compared to healthy controls.^{27,29} These findings suggest the predisposition of subjects with jSLE to develop periodontal disease.

Short-chain fatty acids, such as butyrate, caproate, isocaproate, propionate, isolaverate, and lactate, play an important role in periodontal disorders. They are end-products of bacterial metabolism and have been strongly associated with deep periodontal pockets, loss of attachment, bleeding, and inflammation.⁴² Our study found reduced levels of valerate among patients in the jSLE group. A case-control study showed that caproate, isocaproate/butyrate, isovalerate, isopropanol/methanol, 4-aminobutyrate, choline, sucrose, sucrose-glucose-lysine, lactate-proline, lactate, and proline were increased in patients with periodontal disease.⁴² This could also indicate that decreases in these metabolites are related only to SLE and not to gingivitis, which is probably due to the modification of the inflammatory process due to differences in the characteristics of the biofilm.

Regarding the general health parameters, in the present study, jSLE individuals had increased platelet levels. The literature reported an association between lower levels of taurine and hematologic and immune function disorders, while taurine supplementation or a diet supplementation with taurine improves human health and physical performance.^{33,43} In addition, the jSLE group had lower leukocytes levels

and reduced abundance of glycine. Glycine is a non-essential amino acid produced by the human body. This amino acid is known to decrease activation of inflammatory cells. In addition, the literature reported that a 4-week glycine dietary supplementation can decrease neutrophilic pulmonary infiltration.⁴⁴

The integration of metabolite variations with the identification of the metabolic pathways involved seems to provide a better understanding of the influence of systemic disease on periodontal conditions. Thus, periodontal evaluation and monitoring may, in the future, be part of the short- and long-term disease control measures for SLE patients. Therefore, studies with larger samples that include healthy periodontal and systemic controls are needed to verify what changes are expected on the salivary metabolome due to gingivitis. In addition, longitudinal studies are indicated to monitor the long-term impact of these metabolic changes in the periodontium. Besides, after robust confirmatory studies, complications of jSLE conditions could be monitored by precise knowledge of salivary characteristics as predictors of clinical condition.

Conclusion

Considering the limitations of the present study, it is suggested that jSLE individuals present different salivary metabolic profile compared to controls. The salivary metabolites that differed between the groups were N-acetyl sugars, phenylamine, glycine, taurine, hydroxybutyrate, and valerate. Complications of jSLE conditions could be monitored by proper knowledge of salivary characteristics as predictors of clinical conditions.

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