Reticular and erosive oral lichen planus have a distinct metabolomic profile: A preliminary study using gas chromatography-mass spectrometry

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Background: Oral Lichen Planus is a chronic inflammatory disorder that affects the oral mucosa, with the reticular and erosive forms representing the primary clinical variants of the disease. Previous studies have shown that metabolic alterations may well be involved in the pathogenesis of the disease; however, the molecular mechanisms related to the clinicopathological differences between erosive and reticular forms remain unknown.

Methods: A comparative metabolomic analysis was performed on formalin-fixed and paraffin-embedded tissue samples of erosive (n = 6) and reticular (n = 10) oral lichen planus using gas chromatography-mass spectrometry.

Results: The metabolomic analysis showed a distinct profile between the two clinical variants. Five metabolites (cyclohexanamine, glycine, mannitol/sorbitol, methyl palmitate and trehalose) were significantly diminished in erosive oral lichen planus as compared to the reticular form.

Conclusions: Reticular and erosive forms of oral lichen planus have a distinct metabolic profile. However, further studies using a large number of fresh tissue samples are necessary to confirm this data.

Keywords: autoimmune disease, inflammatory disease, lichen planus, metabolomics, oral lichen planus
TABLE 1 Clinical data of patients with oral lichen planus included in the metabolomic analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Clinical form</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reticular</td>
<td>Male</td>
<td>32</td>
<td>Buccal mucosa</td>
</tr>
<tr>
<td>2</td>
<td>Reticular</td>
<td>Male</td>
<td>56</td>
<td>Buccal mucosa</td>
</tr>
<tr>
<td>3</td>
<td>Reticular</td>
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<td>64</td>
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<tr>
<td>4</td>
<td>Reticular</td>
<td>Female</td>
<td>53</td>
<td>Tongue</td>
</tr>
<tr>
<td>5</td>
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<td>Female</td>
<td>55</td>
<td>Gingiva</td>
</tr>
<tr>
<td>6</td>
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<td>Female</td>
<td>20</td>
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<tr>
<td>7</td>
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</tr>
<tr>
<td>8</td>
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</tr>
<tr>
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<td>Female</td>
<td>64</td>
<td>Tongue</td>
</tr>
<tr>
<td>10</td>
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<td>Male</td>
<td>74</td>
<td>Buccal mucosa</td>
</tr>
<tr>
<td>11</td>
<td>Erosive</td>
<td>Female</td>
<td>61</td>
<td>Buccal mucosa</td>
</tr>
<tr>
<td>12</td>
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<td>Female</td>
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<tr>
<td>16</td>
<td>Erosive</td>
<td>Female</td>
<td>59</td>
<td>Buccal mucosa</td>
</tr>
</tbody>
</table>

an organism or originating from environmental factors.4,5 This tool presents a great potential for the understanding of the mechanisms involved in disease pathogenesis as well as in biomarker discoveries.6 Although metabolomic analyses performed on the saliva,7 urine,8 serum9 and mucosa10 of patients with OLP showed changes in the metabolic profile of OLP, when compared to healthy patients, there is still no consensus about which metabolic pathways are active in this disease and interfere directly in its evolution.

Considering that the differences between the metabolic profile of erosive and reticular OLP remain unclear, this work aims to develop a metabolomic study using gas chromatography-mass spectrometry (GC-MS) in samples of both clinical forms of reticular and erosive OLP. We hypothesize that the metabolic profile varies according to the clinical forms of OLP.

2 MATERIALS AND METHODS

2.1 Samples and subjects

This study was approved by the Research Ethics Committee of the Federal University of Minas Gerais (UFMG) (protocol number: 57753916.3.0000.5149). All the patients agreed to participate and signed a free and informed consent form.

Patients with a clinical and histopathological diagnosis of OLP were divided into two groups: reticular OLP and erosive OLP. Patients who had recently used corticosteroids or who had been diagnosed with other inflammatory disorders, such as erythematous lupus and rheumatoid arthritis, were excluded from this study.

All samples consisted of formalin-fixed paraffin-embedded tissues, and the histopathological diagnosis was confirmed by an oral pathologist.

2.2 Sample preparation

To extract metabolites, one 10 μm-thick tissue section, with an area of 0.3 cm², was collected in microtubes. Extraction was performed as described in prior studies.11

Derivatization was performed by methoximation reaction using 10 μL of methoxyamine in pyridine (15 ppm, 15 mg/mL) (Sigma Aldrich, St Louis, MO). The reaction was carried out overnight at room temperature. For silylation, 10 μL of BSFTA + TMCS (99:1) (Sigma Aldrich) was added, and the reaction was carried out for 1 hour at 70°C. After this period, 100 μL of heptane was added to each sample. Quality controls were prepared by pooling the same amount from each sample and were analysed at regular intervals (after every five samples).

2.3 Gas chromatography-mass spectrometry (GC-MS)

Analyses were performed using a 7890 A Gas Chromatography system coupled to a 5975 C quadrupole Mass Detector (Agilent Technologies®, Santa Clara, CA) equipped with an automatic injector combiPAL autosampler. To perform the analysis, 2 μL of each sample was injected in split mode (ratio 1:10) in a DB-5MS capillary column (30 m in length, 0.25 mm in internal diameter, and 0.25 μm in film thickness) (Agilent Technologies®). Ultrapure helium (99.9999%) was used as carrier gas at a flow rate of 1.0 mL/min. The injector temperature was maintained at 250°C. The oven temperature was programmed at 60°C (1.0 min), with a gradual increase at a rate of 10°C/min up to 325°C. The mass detector was operated in electron impact ionization at 70 eV and in full-scan mode at the range 50-600 m/².

Data processing was performed using XCMS software (version 1.24.1) on the R platform (version 3.2.2).12 Data extraction was performed by the matched filter method. The method parameters were as follows: (a) peak width (fwhm) = 4; (b) signal-to-noise ratio (snthresh) = 1.5; (c) maximum number of peaks per extracted ion chromatogram (max) = 30. Peak grouping was based on a bandwidth correction (bw) = 5 and a width of the overlapped bands of m/z (mzwid) = 0.25. The “fillPeaks” tool was used to remove missing values, and the default “retcor” method was applied with nonlinear alignment and a degree of smoothing for local polynomial regression fitting (span) = 0.5 to correct the retention time. The annotation of metabolites using AMDIS software was search in the Fiehn GC/MS RLT Library in an attempt to identify co-eluted compounds based on the retention index.5 The data set was normalized by the median of intensities before statistical analyses.

2.4 Statistical analysis

Data were Pareto scaled before performing multivariate statistical analysis. Principal Components Analysis and Partial Least Square Discriminant Analysis were carried out using SIMCA 14.1 (Umetrics, Umeå, Sweden). Univariate statistics were calculated using the Statistica.
software, version 10 (StatSoft, Tulsa, OK), applying either the Student’s t test or the Mann-Whitney U test, according to the Kolmogorov-Smirnov, Lilliefors and Shapiro-Wilk normality tests. The threshold for significance was set to $P$-value $<0.05$. Hierarchical clustering analysis was performed using the MetaboAnalyst software, version 4.0.13

FIGURE 1  Principal components analysis score plot built with metabolites identified by gas chromatography coupled to mass spectrometer in the presence of quality controls. Reticular oral lichen planus (OLP) (green circles), Erosive OLP (blue circles) and Quality Controls (red circles). The tight clustering of quality controls observed in this figure confirms that the differences found in the metabolic profile between the studied groups are suggested to be biological. Quality parameters of the model: $R^2$: 0.998 and $Q^2$: 0.564

FIGURE 2  Hierarchical clustering analysis of the metabolic profiles of erosive and reticular oral lichen planus (OLP). The hierarchical clustering analysis was performed using Euclidean distance and complete-linkage in Metaboanalyst 4.0 using the mean ionic abundance of identified metabolites. Reticular OLP samples are demonstrated by green squares and erosive OLP samples by red squares. The hierarchical clustering analysis shows a metabolic distinction of OLP clinical variants, with the exception of one erosive OLP sample and one reticular OLP sample

3 RESULTS

The main clinical data of the subjects included in the study are shown in Table 1. The sample set consisted of ten patients with reticular OLP and six with erosive OLP. The stability of the analytical platform and the reproducibility of the experiments were confirmed by a principal component analysis, which consists of an unsupervised method of multivariate statistics (Figure 1).

A GC-MS analysis enabled the identification of 24 metabolites. Table S1 lists the identified metabolites, along with their respective molecular formulas, monoisotopic mass, and fold changes.

A dendrogram was constructed to highlight the metabolic differences between the clinical forms of OLP (Figure 2). As shown in Figure 2, the clinical variants were placed in different clusters, with the exception of one reticular OLP sample and one erosive OLP sample. To better define the differences in metabolomic profiles between erosive and reticular OLP, a partial least square discriminant
analysis (PLS-DA) model was built. This model also demonstrated a distinct metabolic profile between the clinical forms of OLP. The quality parameters, $R^2_x$: 0.864, $R^2_y$: 0.768 and $Q^2$: 0.602, showed an adequate prediction ability of the model (Figure 3A). To validate the model, a permutation test using 100-iteration was performed (Figure 3B), confirming the lack of overfitting.

Compounds with variable importance in projection (VIP) scores of higher than one were determined in order to identify the compounds that most contributed to the discrimination in the PLS-DA model (Table 2). In addition, the metabolites that presented a $P$-value of less than 0.05 were also determined by univariate analyses, using either Student $t$ or Mann-Whitney U tests. Statistically significant metabolites are listed in Table 2. The levels of cyclohexanamine, glycine, mannitol/sorbitol, methyl palmitate and trehalose were decreased in erosive OLP as compared to the reticular form.

4 | DISCUSSION

Autoimmune diseases consist of a wide range of pathologic conditions that present challenging diagnoses and pathogeneses. The identification of active metabolic pathways through metabolomics has become a fundamental issue in the identification of biomarkers for clinical use. OLP is a relatively prevalent chronic immunoinflammatory disease whose molecular pathogenesis is not fully understood.

Given that OLP can present systemic manifestations, and in some cases even require systemic treatment, previous metabolomic studies have sought to identify potential biomarkers, using serum, urine and saliva samples. However, since metabolic alterations tend to occur initially in the affected tissue, for the study of the pathological changes related to these lesions, samples of the affected mucosa would represent the optimal material. Recently, a tissue-based metabolomics approach of reticular OLP identified 30 potentially dysregulated metabolic pathways, as compared to normal oral mucosa. In the present study, we compared the metabolomic profile of reticular OLP with the erosive form of the disease.

Differences in the abundance of five metabolites when comparing erosive and reticular OLP were observed in our analysis. The differences between erosive and reticular OLP might be greater than only a few metabolites, and different metabolic pathways possibly contribute to the distinct metabolic profiles.
pathways are possibly deregulated in each clinical form. The use of a single analytical platform could be associated with the detection of small differences in the metabolic profile of OLP clinical forms. Moreover, although metabolomics based on formalin-fixed paraffin-embedded tissue is a valuable tool to differentiate biological states, the process of formalin-fixation with paraffin-embedding causes a reduction in several classes of metabolites, such as peptides and steroids. Therefore, future metabolomics analyses of fresh frozen specimens are warranted to complement our findings.

Among the five metabolites that discriminated erosive from reticular OLP, only two, methyl palmitate and glycine, were associated with the immune response.

A decreased abundance of methyl palmitate in erosive OLP was observed. Interestingly, methyl palmitate is a fatty acid ester known as an oxidative stress and inflammatory response inhibitor by decreasing the expression of nuclear factor kappa-B (NF-κB). NF-κB induces proinflammatory cytokines and chemokines, which mediate the recruitment of immune cells, T-lymphocyte activation, and the establishment of inflammation. In addition, this nuclear factor is involved in the pathogenesis of OLP and other autoimmune diseases, such as rheumatoid arthritis, systemic diseases, lupus erythematosus, type 1 diabetes, multiple sclerosis and inflammatory bowel disease. Regarding OLP, studies have described an activation of NF-kB in both erosive and reticular forms. Glycine is an amino acid that plays a relevant role in blocking inflammation and cytoprotection. In OLP, apoptosis of basal and parabasal epithelial cells represents a pivotal event in the pathogenesis of the disease. The erosive form of OLP presents a thinner epithelium with a higher apoptotic index when compared to the reticular form. In our study, the metabolic evaluation showed reduced levels of glycine in the erosive OLP, which is in line with the increased apoptotic activity and the inflammatory state in this form of the disease. However, further studies are still necessary to show a cause-effect relationship between them, and functional analyses are needed to determine the proper role of glycine in the pathogenesis of OLP.

A major limitation of the present study is related to the small sample amount available for analysis. Moreover, despite selecting representative samples for analysis, molecular heterogeneity related to the inflammatory reaction is expected to occur. This study should be considered as a pilot for future investigations with a larger sample size and employing a comprehensive metabolomic analysis through multi-analytical platforms, using different combinations of extraction solvents and including fresh tissue samples.

In conclusion, differences in the levels of metabolites when comparing erosive and reticular OLP were found, but further studies using a large number of fresh tissue samples are necessary to confirm this data.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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REFERENCES


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