The impact of chronic gingivitis management on the cytokine and anti-PPAD expressions in juvenile systemic lupus erythematosus. A six-month follow-up.

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Abstract

Objective: Evaluate how chronic gingivitis treatment impacts the oral and circulating cytokine expressions after six-month follow-up in patients with Juvenile systemic lupus erythematosus (JSLE). Besides, also evaluate the circulating expression of anti-\textit{Porphyromonas gingivalis} peptidylarginine deiminase antibodies (anti-PPAD) before and after treatment. Background: JSLE patients present a worse periodontal condition associated with higher gingival crevicular fluid (GCF) levels of interleukin (IL)-1β, IL-8, granulocyte colony-stimulating factor (G-CSF), interferon-γ and monocyte chemoattractant protein (MCP)-1. Material and Methods: Twenty-one adolescents with JSLE (mean age: 16.2 ± 1.5 years) were recruited. Participants were rheumatologically and periodontally examined. All individuals were clinically diagnosed with gingival inflammation. Chronic gingivitis treatment consisted of supragingival scaling, prophylaxis, and oral hygiene instructions. The cytokine levels were determined by bead-based multiplex assays and the anti-PPAD levels by ELISA. Gingival crevicular fluid (GCF) and serum samples were collected at baseline and 6 months after treatment. Results: We observed a reduction in attachment loss, SLE Disease Activity Index (SLEDAI), IL-1β, IL-10 and MCP-1 GCF levels, and the IL-4 and IL-5 serum levels six months after periodontal treatment. On the other hand, a significant increase in GCF expression of IL-4, IL-12, IL-17, IFN-γ and serum levels of anti-PPAD antibody was observed. Conclusion: JSLE patients seem to positively benefit from periodontal treatment by a significantly reduced CAL, a GCF reduction of pro-inflammatory cytokines and an increasing of anti-inflammatory ones. However, an increase in the GCF expression of IL-17 and the serum expression of anti-PPAD antibody 6 months after periodontal treatment might negatively affect the treatment outcome of such patients in the long term.
Introduction

Juvenile-onset systemic lupus erythematosus (jSLE) is a rare but severe autoimmune disease that can cause significant damage, disability and/or death. Its onset occurs before the age of 18 and affects approximately 20% of SLE patients. According to Charras et al., jSLE is more aggressive than adult-onset SLE, with higher disease activity and medication burden. Both can contribute to an increased morbidity and mortality, more severe organ manifestations and the presence of more significant damage at the time of diagnosis. jSLE management is complex due to the heterogeneity between individuals in disease presentation and progression, treatment response, and overall disease severity.

The pathogenesis of jSLE is associated with an imbalance between IL-17A expressing effector vs regulatory T cells, creating a pro-inflammatory profile where uncontrolled IL-17A production contributes to immune cell activation and infiltration of inflamed tissues.

A systematic review & meta-analysis has shown that adult patients with SLE have an increased risk of developing periodontitis. Recently, our group has shown that patients with jSLE present a worse periodontal condition associated with higher gingival crevicular fluid (GCF) levels of interleukin (IL)-1β, IL-8, granulocyte colony-stimulating factor (G-CSF), interferon-γ and monocyte chemoattractant protein (MCP)-1 when compared with healthy controls. Herein, we investigated whether chronic gingivitis management could prevent periodontal disease progression and reduce the oral expression of pro-inflammatory cytokines in jSLE patients, which could reduce the risk of developing periodontitis in adulthood.

Besides the immunoregulatory impact caused by the imbalance of cytokine expression, a periodontal bacterium named Porphyromonas gingivalis has been associated with autoimmunity. P. gingivalis presents a specific virulence factor, the peptidyl-arginine deiminase (PAD) enzyme, called P. gingivalis PAD (PPAD), which is similar to the PAD
enzyme found in the cytoplasm of various cell types. Our group has reported the presence of anti-PPAD antibodies in serum from both jSLE patients and controls. PPAD catalyses the citrullination reaction, which can lead to the formation of citrullinated peptides, against which the immune system develops a humoral response, with the formation of antibodies, including anti-PPAD. Chronic exposure to these citrullinated proteins in predisposed patients can lead to loss of immune tolerance and the formation of an autoimmune response.

Therefore, we aimed to evaluate how chronic gingivitis treatment impacts the oral and circulating cytokine expression after six months of follow-up. Besides, we also evaluated the circulating expression of anti-

Porphyromonas gingivalis

peptidylarginine deiminase antibodies (anti-PPAD) before and after treatment.

Materials and methods

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

Study population

This longitudinal study enrolled twenty-one patients (1 man and 20 women; age range: 13 to 20 years) diagnosed with jSLE according to the American College of Rheumatology. All subjects with jSLE underwent treatment at the Center for Adolescent Health Studies (NESA) at the pediatric rheumatology clinic, University Hospital Pedro Ernesto (HUPE), Rio de Janeiro, Brazil.
All subjects presented at least 20 natural teeth (excluding the 3rd molars) and had been diagnosed with gingivitis according to the American Academy of Periodontology (probing depth (PD) ≤ 3 mm and clinical attachment level (CAL) ≤ 1 mm with bleeding on probing (BOP)). PD of 4 mm was tolerated in cases of gingival hyperplasia, on all sites, and CAL up to 3 mm in cases of mechanical trauma such as brushing on buccal and/or lingual/palatine faces.

Exclusion criteria for both groups were: pregnancy, lactation or periodontal/orthodontic treatments in the last 6 months. Medications including antibiotics were not used as exclusion criteria as jSLE patients regularly use antibiotics due to the high risk of infections.

**Clinical examinations**

All participants went through anamnesis and clinical examination where general medical and dental data, information on jSLE disease history, medications used, and other co-existent systemic diseases were collected. A rheumatologist (FRS) assessed disease activity using the SLE disease activity index (SLEDAI) and cumulative damage using the Systemic Lupus International Collaborating Clinics index (SLICC).

Two calibrated periodontists (MRCS and JCC) performed all the clinical dental examinations at the Faculty of Odontology, UERJ, Rio de Janeiro, Brazil. A periodontal probe was used for the periodontal assessment (Hu-Friedy, Chicago, IL, USA) in six sites per tooth. The following parameters were determined: PD, CAL, BOP and percentage of plaque formation. The intra-examiner agreement value was 90%, and the inter-examiner was 70% for PD and CAL variables, accepting a ± 1 mm variable.

**Chronic gingivitis treatment**
Chronic gingivitis treatment consisted of oral hygiene instructions, supragingival scaling, prophylaxis, and topical fluoride application. The treatment was carried out on average in a session of 40 minutes. Supragingival scaling was performed with Gracey and McCall curettes (HU-Friedy®, Chicago, Illinois, USA) and with a portable ultrasound device (Cavitron® Select SPS, Dentsply Sirona, France) by one of the two operators (MRCS, JCC). The reassessment of patients was carried out six months after the end of periodontal treatment.

**Sample collection**

Serum and GCF samples were collected in conjunction with the dental examination. GCF samples were collected and pooled from three to four sites from different teeth, selected as the most inflamed sites with clinical signs of redness or oedema in the gingiva from each patient. GCF was collected by a washing method where each site was washed with 0.5 ml phosphate-buffered saline during continuous aspiration. Peripheral blood was collected by venous puncture using Vacutainer tubes (BD Vacutainer, Franklin Lakes, NJ, USA).

**Basic laboratory measurements**

Routine laboratory procedures performed blood counts of total leukocytes, neutrophils, monocytes, and lymphocytes, and urea and creatinine concentrations were measured (data missing from n=1-3 baseline and n=1-5 after treatment). Erythrocyte sedimentation rate (ERS), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were also determined (data missing from n=1-5 baseline and n=1-5 after treatment).

**Multiplex assay**
The levels of G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, MCP-1, MIP-1β, and TNF-α in serum and GCF were determined using a commercially available multiplex bead immunoassay kit (Bio-Rad Laboratories, Hercules, CA) in a multiplex analyser (Bio-plex 200®, Bio-Rad Laboratories, Hercules, CA) according to the manufacturer instructions. Fifty microliters of the homogenised samples were analysed, and the results were presented as total amounts (picogram). Cytokines with detectability ≥ 30% in each fluid were included in the analysis.

ELISA assay

For the detection of serum anti-PPAD antibodies, a microtiter plate was coated with 0.1µg/ml of PPAD peptide (CLGTDALHC-Cit-THEVADKGC; Aminotech Pesquisa e Desenvolvimento, São Paulo, Brazil) diluted in 0.1 M coating buffer solution (pH 9.5; BD OptEIATM, BD Biosciences, San Jose, USA) overnight at 37°C. The plate was protected to prevent light sensitisation. The plate was washed three times with PBS containing 0.05% Tween 20 (PBS-T) (Sigma-Aldrich, Saint Louis, USA) and then blocked with 1% bovine serum albumin solution (BSA) in PBS-T at room temperature for 2 hours. Next, the plate was incubated with serum at 1:500 dilution in PBS for 30 minutes at room temperature and washed again as above. Then specific Fc Biotinylated Human Anti-IgG monoclonal antibody (1:1000; Sigma-Aldrich, Saint Louis, USA) was added and incubated for 1 hour at room temperature. After washing, streptavidin-conjugated with horseradish peroxidase enzyme (1:1000; BD Biosciences, San Jose, USA) was added for 1h at 37°C. The plate was rewashed, and hydrogen peroxide and 3,3', 5,5' tetramethylbenzidine chromogenic substance (TMB) (BD Biosciences, San Jose, USA) was added to each well. After 2 min, the reaction was
stopped using the phosphoric acid solution (BD OptEIATM (BD Biosciences, San Jose, USA). The plate was then read in a spectrophotometric at a wavelength of 450nm (Microplate reader, TP-reader® - Thermo Plate, China). Serum levels of anti-PPAD antibodies were expressed in ELISA units (EU).

**Statistical analysis**

Data analysis was performed using SPSS 21.0 (IBM, Chicago, USA). The normality of data was checked with the Kolmogorov-Smirnov test. Continuous variables are presented as median (interquartile range) and categorical variables as percentages. Age is presented as mean (standard deviation). The McNemar test was used for qualitative data and the Wilcoxon test for numerical data between baseline and after treatment. The level of significance was set at 5%. Spearman's rank correlation coefficient was determined by the correlation between clinical and immunological variables, and only $r \geq 0.6$ and $p \leq 0.01$ was considered significant.

**Results**

*Clinical and demographic analysis*

We observed a significant reduction of SLEDAI followed by a significantly increased immunosuppressive intake during the 6-month follow-up. The demographic and serological data are presented in Table 1. Chronic gingivitis treatment significantly reduced the CAL, but no significant differences were observed for the PD and % plaque. There was a tendency towards an increase in % BOP levels after treatment (Table 2).

*Cytokine expression and anti-PPAD*
Out of 17 measured cytokines, 15 and 13 cytokines were detected in more than 30% of the GCF and serum samples, respectively (Figure 1A and B, respectively). The GCF expression of IL-1β, IL-10 and MCP-1 reduced significantly in jSLE patients after 6-months. On the other hand, IL-4, IL-12, IL-17, GM-CSF, and IFN-γ significantly increased in such patients (Figure 2A). In serum, there was a significant reduction of IL-4 and IL-5 expression at the follow-up (Figure 2B). No significant changes were observed for the remaining cytokines. We observed a significant increase in anti-PPAD serum expression after a 6-month follow-up period (Figure 3).

Correlations

There was no significant correlation between the biomarkers in GCF or serum with periodontal clinical parameters. Moreover, there was no significant correlation between serum and GCF expressions. On the other hand, significant correlations were observed between biomarkers in serum and GCF separately. The correlation heat map is presented in Figure 4.

Discussion

Herein, we showed that chronic gingivitis treatment might help control periodontal disease progression in jSLE patients by significantly reducing the clinical attachment level and keeping pocket depth stable after 6 months. Our group has previously shown that jSLE patients presented significantly higher percentages of both dental plaque and bleeding and increased mean PD and CAL when compared to systemically healthy controls.\(^5\) Thus, chronic gingivitis management could wisely be recommended to avoid oral health deterioration in jSLE patients. Besides, we reported a significant reduction in
GCF expression of IL-1β, IL-10, and MIP-1β. Our group has previously shown that jSLE patients exhibited significantly higher GCF levels of IL-1β compared with the controls.\textsuperscript{5} IL-1β is strongly associated with periodontal disease severity.\textsuperscript{15,16} Moreover, periodontal treatment has been shown to significantly reduce the IL-1β GCF levels associated with periodontal clinical improvements.\textsuperscript{17} Thus, the present study suggests that jSLE response to periodontal therapy might be similar to systemically healthy patients.

Besides, we observed a serum reduction of IL-4 and IL-5 expression associated with SLEDAI reduction after 6-month follow-up. Our findings are partially supported by Fabbri et al. \textsuperscript{18}, who reported a significant improvement in SLEDAI after periodontal treatment in a 3-month follow-up study. Moreover, IL-4 and IL-5 have been reported to have an essential role in the SLE pathogenesis.\textsuperscript{19,20} Mahmoudi et al. \textsuperscript{21} reported that IL-4 gene variants were associated with jSLE and might have a role in the pathophysiology of the disease. Our serum IL-4 results suggested that the combination of chronic gingivitis treatment and systemic disease management might help to control the Th2 overexpression observed in jSLE. However, this speculation is limited due to the absence of a control group of jSLE patients without periodontal treatment and the increased intake of immunosuppressant medication, which might have a significant impact on the serum changes herein observed.

The patients included in this study were treated with corticosteroids (Prednisone), immunosuppressives (Azathioprine) and immunomodulators (Reuquinol/hydroxychloroquine). Immunosuppressive drugs such as hydroxychloroquine and prednisone have been reported to inhibit Th-related cytokines and IL-4 production in the blood of patients with SLE,\textsuperscript{22,23} which might explain the reduced serum levels of IL-4 after treatment. Thus, further studies assessing the impact of immunomodulators in periodontal disease progression and its potential as adjuncts to
conventional local disinfection treatments may bring benefits and deserve to be further explored.\textsuperscript{24}

Although the jSLE patients responded well to the periodontal treatment, we observed that the percentage of plaque formation and gingival bleeding bounced back to baseline levels after 6 months. A significant increase in the IL-17 expression in GCF was also observed. The increased inflammation after treatment might have induced a massive neutrophils/macrophages migration to the gingival area. As a consequence, upregulation of IL-17 occurred. IL-17 shown to be a critical driving force of inflammatory bone loss in animal models of periodontitis.\textsuperscript{25} An up-regulation of IL-17 can induce osteoclastogenic mediators' production and promote the expression of RANKL, therefore having a destructive role in the pathogenesis of periodontal disease.\textsuperscript{26} Taken together, those markers could increase the risk of periodontal disease progression. \textbf{We strongly believe that the interval between supportive periodontal care appointments should be kept no longer than 6 months to prevent disease progression.}

Our present data showed that the periodontal treatment provoked a systemic response to PPAD, increasing the circulating expression of PPAD antibodies in jSLE patients. Our group has been the first to report the presence of anti-PPAD antibodies in serum from jSLE patients.\textsuperscript{9} However, the long-term implications of such findings in jSLE patients are unknown. On the other hand, PPAD has been strongly investigated in patients with rheumatoid arthritis (RA). Kobayashi et al.\textsuperscript{27} reported that serum PPAD IgG levels might affect the clinical response to the biological disease-modifying anti-rheumatic drug in patients with rheumatoid arthritis. Shimada et al.\textsuperscript{28} have shown that supragingival scaling failed to decrease the serum levels of anti-PPAD IgG and suggested a role for PPAD in protein citrullination in patients with rheumatoid arthritis and periodontitis. It is possible
to speculate that an adverse effect might also be observed in other autoimmune diseases, such as SLE.

A critical limitation of the present study is the absence of a control group with jSLE and no periodontal disease. This study would benefit significantly with the addition of two control groups; one composed of systemically healthy patients with gingivitis and another having jSLE patients with clinically healthy periodontium. Such groups could help clarify whether the present finds are associated with the proposal periodontal disease treatment or merely a consequence of jSLE disease management. Besides, there was a significant increase in immunosuppressive use, which substantially affected the SLEDAI and cytokines expressions. On the other hand, our study is the first to shed light on the management of periodontal disease in jSLE patients to prevent future oral complications that could lead to tooth loss-associated local and systemic complications in such patients.

**In conclusion**, jSLE patients seem to positively benefit from periodontal treatment by a significantly reduced CAL, a GCF reduction of pro-inflammatory cytokines and an increasing of anti-inflammatory ones. However, an increase in the GCF expression of IL-17 and the serum expression of anti-PPAD antibody 6 months after periodontal treatment might negatively affect the treatment outcome of such patients in the long term.

**Funding**

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**Conflict of interest**

The authors declare that there is no conflict of interest.
References


Table 1 – Demographic, clinical and serological characteristics of participants with juvenile systemic lupus erythematosus (jSLE) at baseline and after treatment.

<table>
<thead>
<tr>
<th></th>
<th>jSLE (baseline)</th>
<th>P</th>
<th>jSLE (6-months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLEDAI median (ir)</td>
<td>6 (9.5)</td>
<td>0.01</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Moderate/severe activity n (%)</td>
<td>12 (57.1)</td>
<td>0.04</td>
<td>4 (19.1)</td>
</tr>
<tr>
<td>No activity/mild activity n (%)</td>
<td>9 (42.9)</td>
<td></td>
<td>17 (80.9)</td>
</tr>
<tr>
<td>SLICC with damage (%)</td>
<td>6 (28.6)</td>
<td></td>
<td>6 (28.6)</td>
</tr>
<tr>
<td>Use of Corticosteroid, n (%)</td>
<td>15 (71.4)</td>
<td>0.07</td>
<td>9 (42.8)</td>
</tr>
<tr>
<td>Use of Immunosuppressive, n (%)</td>
<td>11 (52.4)</td>
<td>0.03</td>
<td>17 (80.9)</td>
</tr>
<tr>
<td>Use of antimalarial, n (%)</td>
<td>16 (76.2)</td>
<td>0.2</td>
<td>19 (90.9)</td>
</tr>
<tr>
<td>Total leukocytes (10$^3$/mm), median (ir)</td>
<td>4.4 (0.65)</td>
<td>0.63</td>
<td>4.4 (0.5)</td>
</tr>
<tr>
<td>Neutrophils count (%), median (ir)</td>
<td>59 (12.3)</td>
<td>0.72</td>
<td>53.5 (13)</td>
</tr>
<tr>
<td>Monocytes count (%), median (ir)</td>
<td>7.0 (4)</td>
<td>0.43</td>
<td>9 (4.5)</td>
</tr>
<tr>
<td>Lymphocytes count (%), median (ir)</td>
<td>31 (8)</td>
<td>0.49</td>
<td>34 (19)</td>
</tr>
<tr>
<td>ESR (mm/h), median (ir)</td>
<td>17.5 (38)</td>
<td>0.46</td>
<td>23 (29)</td>
</tr>
<tr>
<td>Urea (mg/dL), median (ir)</td>
<td>23 (9)</td>
<td>0.21</td>
<td>19 (12)</td>
</tr>
<tr>
<td>Creatinine (mg/dL), median (ir)</td>
<td>0.7 (0.2)</td>
<td>0.28</td>
<td>0.7 (0.2)</td>
</tr>
<tr>
<td>GOT (IU/L), median (ir)</td>
<td>18 (5)</td>
<td>0.24</td>
<td>20 (9)</td>
</tr>
<tr>
<td>GPT (IU/L), median (ir)</td>
<td>10.5 (9)</td>
<td>0.43</td>
<td>11 (14)</td>
</tr>
</tbody>
</table>

Table 2. Median (interquartile range) of the periodontal clinical parameters of juvenile systemic lupus erythematosus (JSLE) patients at baseline and after treatment.

<table>
<thead>
<tr>
<th>Variables</th>
<th>JSLE (baseline)</th>
<th></th>
<th></th>
<th>JSLE (6-months)</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Median</td>
<td>IR</td>
<td>p</td>
<td>Median</td>
<td>IR</td>
</tr>
<tr>
<td>%PD ≤ 3 mm</td>
<td>99.4</td>
<td>3.3</td>
<td>0.3</td>
<td>98.5</td>
<td>4.0</td>
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<td>%PD 4-5 mm</td>
<td>0.6</td>
<td>2.9</td>
<td>1</td>
<td>0.6</td>
<td>2.9</td>
</tr>
<tr>
<td>PD (mean)</td>
<td>2.3</td>
<td>0.4</td>
<td>0.08</td>
<td>2.3</td>
<td>0.2</td>
</tr>
<tr>
<td>%CAL 0</td>
<td>94.6</td>
<td>8.8</td>
<td>0.004</td>
<td>98.8</td>
<td>3.6</td>
</tr>
<tr>
<td>%CAL 1-2 mm</td>
<td>4.2</td>
<td>7.1</td>
<td>0.03</td>
<td>1.2</td>
<td>3.6</td>
</tr>
<tr>
<td>%CAL 3-4 mm</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CAL (mean)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.01</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>%Plaque</td>
<td>69.6</td>
<td>37.3</td>
<td>0.1</td>
<td>58.9</td>
<td>33.5</td>
</tr>
<tr>
<td>%BOP</td>
<td>15.2</td>
<td>17.0</td>
<td>0.077</td>
<td>24.1</td>
<td>12.9</td>
</tr>
</tbody>
</table>

Legend: PD: probing depth; CAL: clinical attachment level; BOP: bleeding on probing. P-value: comparison between before and after treatment in JSLE patients. p: comparison between before and after treatment in JSLE patients (n=21).
Figure 1B. Cytokine detectability in gingival crevicular fluid (A) and serum (B) from patients with juvenile systemic lupus erythematosus at baseline and 6 months after treatment (n=21). Detectability is presented in percentage. GCF: gingival crevicular fluid; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN: interferon; IL: interleukin; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein; TNF: tumour necrosis factor.

Figure 1. Cytokine detectability in gingival crevicular fluid (A) and serum (B) from patients with juvenile systemic lupus erythematosus at baseline and 6 months after treatment (n=21). Detectability is presented in percentage. GCF: gingival crevicular fluid; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN: interferon; IL: interleukin; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein; TNF: tumour necrosis factor.
Figure 2. Cytokine levels (pg/mL) in gingival crevicular fluid (A) and serum (B) from patients with juvenile systemic lupus erythematosus at baseline and 6 months after treatment (n=21). Cytokines with less than 30% of detectability were excluded from the figure. Comparisons were assessed by Wilcoxon matched-pair signed-rank test. GCF: gingival crevicular fluid; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN: interferon; IL: interleukin; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein; TNF: tumour necrosis factor.
**Figure 3.** There were serum levels of anti-PPAD in ELISA units from patients with juvenile systemic lupus erythematosus at baseline and 6 months after treatment (n=21). Comparisons were assessed by Wilcoxon matched-pair signed-rank test. PPAD: *Porphyromonas gingivalis* peptidylarginine deiminase.
Figure 4. Correlation heat maps of cytokines in gingival crevicular fluid and serum of patients with juvenile systemic lupus erythematosus at baseline and 6 months after treatment (n=21). The correlation between serum levels of anti-PPAD and cytokines was also assessed. Heat maps depict correlation coefficients between the molecules. Spearman’s rank correlation coefficient determined correlations between immunological variables, and only $R \geq 0.6$ and $p \leq 0.01$ was considered significant (*). GCF: gingival crevicular fluid; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN: interferon; IL: interleukin; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein; PPAD: *Porphyromonas gingivalis* peptidylarginine deiminase.