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Figueredo, CM, Martins, AP, Lira-Junior, R, Menegat, JB, Carvalho, AT, Fischer, RG, Gustafsson, A

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## **Activity of inflammatory bowel disease influences the expression of cytokines in gingival tissue**

Carlos Marcelo Figueredo<sup>1</sup>, Ana Paula Martins<sup>1</sup>, Ronaldo Lira-Junior<sup>1</sup>, Juliana Bittencourt Menegat<sup>1</sup>, Ana Teresa Carvalho<sup>2</sup>, Ricardo Guimarães Fischer<sup>1</sup>, Anders Gustafsson<sup>3</sup>

<sup>1</sup>Department of Periodontology, Faculty of Odontology, Rio de Janeiro State University, Rio de Janeiro, Brazil

<sup>2</sup>Department of Internal Medicine, Faculty of Medicine, Rio de Janeiro State University, Rio de Janeiro, Brazil

<sup>3</sup>Division of Periodontology, Department of Dental Medicine, Karolinska Institutet, Stockholm, Sweden

### **Corresponding author:**

Carlos Marcelo Figueredo

Rio de Janeiro State University, Faculty of Odontology, Boulevard 28 de Setembro 157, 2º Andar, Vila Isabel, Rio de Janeiro, RJ 20551-030, Brazil.

Phone: +55 21 28688282

E-mail address: cmfigueredo@hotmail.com

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## ABSTRACT

**Objective:** To assess the cytokine expression in gingival and intestinal tissue from periodontitis patients with IBD and evaluated if IBD activity is a covariate to the amount of gingival cytokines.

**Design:** This cross-sectional study included 21 patients with periodontitis and IBD. Gingival and intestinal tissues were collected from each patient and homogenised using a cell disruptor. Cytokine expression (IL-1 $\beta$ , IL-4, IL-6, IL-10, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IL-17A, IL-17F, IFN- $\gamma$ , sCD40L, and TNF- $\alpha$ ) was evaluated using bead-based multiplex technology. An inflammation score was developed using the intestinal cytokines that showed good accuracy to discriminate IBD active patients from those in remission and then a similar score was applied to gingival tissue.

**Results:** IL-4, IL-10 and IL-21 expressions were significantly increased in gingival tissue from patients with an active disease as compared to those with a disease in remission. The inflammation score (mean value of IL-1 $\beta$ , IL-6, IL-21, and sCD40L) was significantly higher in gingival tissue from patients with IBD activity. There was a significant correlation between gingival and intestinal inflammation scores ( $\rho=0.548$ ;  $p=0.01$ ). Significantly higher IL-23 and IFN- $\gamma$  levels and lower IL-31 and TNF- $\alpha$  levels were observed in gingival tissues than in intestinal ones.

**Conclusion:** Activity of inflammatory bowel disease influenced the cytokine expression in gingival tissue, as observed by increased levels of IL-4, IL-10 and IL-21, and a higher inflammation score in patients with active disease.

**Keywords:** periodontitis, inflammatory bowel disease, cytokines

## **SUMMARY BOX**

### **What is already known about this subject?**

- The prevalence of periodontitis is increased in patients with IBD and cytokines are relevant to the pathogenesis of both diseases.
- The activity of IBD influences the inflammatory response in other compartments besides the intestine, but its impact in the mouth's inflammation is unknown.

### **What are the new findings?**

- Activity of IBD increased the expression of IL-4, IL-10 and IL-21 in gingival tissue.
- An inflammation score was higher in gingival tissue from patients with active IBD.
- There was a correlation between an inflammation score in gingival and intestinal tissues.

### **How might it impact on clinical practice in the foreseeable future?**

- The finding that activity of IBD affects gingival tissue inflammation impacts on the decision of controlling periodontal lesions in patients having both diseases. Patients with IBD should be evaluated to the presence of inflammation in gingiva, aiming to prevent considerable tissue destruction around teeth during the activity period of IBD.

## INTRODUCTION

Periodontal disease is a biofilm-induced chronic inflammatory disease that affects tooth-supporting tissues. Its severe form, which can lead to tooth loss, affects over 740 million people worldwide[1]. Similar to the pathogenesis of inflammatory bowel disease (IBD), periodontal disease results from a complex interplay between the microbiota and the host immune-inflammatory response, and is influenced by genetic and environmental factors. Although the microbiota is a requisite, the persistence and dysregulation of the host immune and inflammatory responses are mainly responsible for the destruction of the periodontal tissues[2, 3].

Studies have shown that IBD patients present an increased prevalence of periodontal disease[4, 5], as well as a higher severity and a greater extent of periodontitis[5], but the mechanisms responsible for this elevation are not well understood. As the development of both diseases is related to an aberrant immune response, the inflammatory response might be a link between them. Indeed, our group has previously found higher IL-18 levels in serum from patients with IBD and periodontitis[6]. Nevertheless, an important point that has not yet been addressed is whether or not the activity of IBD could play a role in the inflammatory response in periodontal tissues.

In fact, the activity of IBD influences the inflammatory response not only in the intestinal tissue[7], but also in the blood[8, 9]. Globig *et al.* have shown an enrichment of Th17 and Treg cells in active lesions in patients with both Crohn's disease (CD) and ulcerative colitis (UC) compared with quiescent/mildly inflamed lesions[10]. Also, positive correlations have been found between disease activity scores and mRNA levels of IL-17A, IL-23, and IL-6 in patients with CD and UC[11]. A similar pattern is observed in sera, where increased levels of TNF- $\alpha$  have been recorded in patients with active UC in comparison to patients with inactive disease[8]. With that in mind, we hypothesise that the intestinal activity of IBD may influence the cytokine expression in gingival tissue. As a consequence of the high inflammatory activity, bone degradation, a hallmark of periodontitis, could be induced[12].

IL-1 $\beta$ , IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, INF- $\gamma$ , sCD40L, and TNF- $\alpha$  are all potentially relevant cytokines in the Th17 response, which plays a significant role in both periodontitis[13] and IBD[10]. Therefore, this study aimed to assess cytokine expression in gingival and intestinal tissue from

periodontitis patients with IBD and evaluate whether or not gingival cytokines covariate according to IBD activity.

## **MATERIAL AND METHODS**

### **Patients and clinical assessment**

This cross-sectional study selected 21 patients (17 women and 4 men; mean age  $40.52 \pm 14.77$  years) with IBD and chronic periodontitis from the outpatient clinic of the Department of Gastroenterology at the Pedro Ernesto University Hospital – Rio de Janeiro State University. Ten patients were diagnosed with CD and 11 patients with UC. The diagnoses were made according to well-established clinical, endoscopic, radiologic, and histological parameters. Chronic periodontitis was defined using the definition established by the American Academy of Periodontology[14]. Patients diagnosed with CD or UC were included in the study if they had at least 10 teeth with probing depth (PD)  $\geq 5$  mm and clinical attachment loss (CAL)  $\geq 4$  mm in at least 4 sites, in different teeth. Patients were excluded if they had previously undergone non-surgical periodontal treatment or used antibiotics in the 6 months preceding their enrolment in the study. Pregnant and breastfeeding women were also excluded. This study was approved by the Research Ethics Committee and patients gave their written informed consent.

IBD activity was evaluated clinically and laboratory employing the Harvey-Bradshaw index[15] for CD and the Truelove and Witts score[16] for UC. Patients were taking the following medications: mesalazine (n=7), mesalazine plus azathioprine (n=8), mesalazine, azathioprine plus TNF- $\alpha$  inhibitor (n=4) and mesalazine plus steroid (n=2).

A calibrated examiner performed a comprehensive periodontal examination using a manual probe (UNC-15, Hu-Friedy Manufacturing Company, Inc., Chicago, IL, USA). The examination included assessment of PD, CAL, bleeding on probing (BoP) and visible plaque index (VPI). PD and CAL measurements were determined at six sites per tooth. BOP and VPI were determined at four sites per tooth, excluding third molars. Intra-examiner concordance was 95% for both PD and CAL within 1 mm.

### **Tissue collection**

This study involved 42 tissue specimens from 21 patients. Gingival and intestinal tissues were always paired and collected no longer than seven days apart. Gingival tissue

was collected from inflamed sites, after anesthesia, with a punch of 1.5 mm positioned towards the periodontal pocket bottom. Sites for collection had PD  $\geq$  4 mm and CAL  $\geq$  3mm. Intestinal biopsy was collected from the most inflamed area and performed at the time of colonoscopy. After collection, tissues were washed with phosphate buffered saline (PBS, Sigma-Aldrich St-Louis, USA) and stored at -70° C until homogenisation.

Tissue was weighed on an analytical balance (Ohaus, Parisppany, USA) and then transferred to a microtube containing ultrapure 3.0 mm zirconia beads, PBS and protease inhibitor (Sigma-Aldrich, St. Louis, USA). Tissue homogenisation was performed using a cell disruptor (Loccus Biotechnology, Brazil) at a speed of 4,000 rpm. After homogenisation, homogenate was collected and centrifuged at 10,000 rpm for 10 minutes. The supernatant was then stored at -70° C until analysis.

### **Multiplex assay**

The expression of fifteen cytokines (IL-1 $\beta$ , IL-4, IL-6, IL-10, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IL-17A, IL-17F, IFN- $\gamma$ , sCD40L, and TNF- $\alpha$ ) was evaluated using a bead-based multiplex immunoassay. Fifty microliters of the homogenised samples were analysed using a commercially available kit (Bio-Rad Laboratories, Hercules, CA, USA) in a multiplex analyser (Bio-plex 200, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The concentrations of the unknown samples were calculated from the standard curve using the Bio-Plex Manager Software (Bio-Rad Laboratories, Hercules, CA, USA). Cytokine levels were adjusted for biopsy weight and were presented as picogram/milligram of tissue (pg/mg).

### **Inflammation score**

Firstly, we selected the intestinal cytokines that showed a significant difference between disease activity and remission. Then, the area under the curve was calculated for these cytokines and, in order to develop an inflammation score, we selected those with good accuracy ( $>.80$ ) in discriminating between activity and remission in intestinal tissue. Four cytokines showed good accuracy (IL-1 $\beta$ , IL-6, IL-21, and sCD40L) and were chosen to constitute the score. The score was determined as the mean value of the four cytokines. After the score was established, it was also calculated as the mean value of the four gingival cytokines.

## **Statistical analysis**

Data analysis was performed using SPSS 20.0 (IBM, Chicago, USA). Cytokine data are presented as median and interquartile range. Wilcoxon test was used to compare the cytokine levels in gingival and intestinal tissues. Mann-Whitney and Fisher exact test were used, when indicated, to compare cytokine expression between IBD activity and remission. Spearman's rank correlation coefficient ( $\rho$ ) was used for the correlation analysis. Differences were considered to be statistically significant at  $p \leq 0.05$ . A cytokine network was built based on the significant correlation coefficients ( $\rho > 0.5$ ;  $p < 0.05$ ) between cytokine pairs for gingival and intestinal tissues, using the `nwcommands` (<http://nnwcommands.org>).

## **RESULTS**

This study enrolled 21 patients with IBD and periodontitis, 8 in IBD activity and 13 in remission. All patients were diagnosed with moderate to severe chronic periodontitis. There were no significant differences in demographic and periodontal parameters between patients with active IBD and those in remission. (Table 1).

**Table 1.** Demographic and periodontal data of patients with active IBD and those in remission.

<b>Variable</b>	<b>IBD activity (n=8)</b>	<b>IBD remission (n=13)</b>	<b>Whole sample (n=21)</b>
<b>Age (years)</b>	38.87 ( $\pm$ 14.12)	41.53 ( $\pm$ 15.63)	40.52 ( $\pm$ 14.77)
<b>Gender (male/female)</b>	-	-	4/17
<b>Crohn's disease (n)</b>	4	6	10
<b>Ulcerative colitis (n)</b>	4	7	11
<b>VPI (%)</b>	27.87 ( $\pm$ 21.94)	45.00 ( $\pm$ 24.66)	38.1 ( $\pm$ 24.03)
<b>BoP (%)</b>	27.37 ( $\pm$ 21.08)	39.53 ( $\pm$ 23.09)	34.90 ( $\pm$ 22.08)
<b>PD <math>\leq</math> 3 mm (%)</b>	85.12 ( $\pm$ 9.80)	81.23 ( $\pm$ 9.93)	82.68 ( $\pm$ 9.59)
<b>PD 4-6 mm (%)</b>	14.37 ( $\pm$ 8.99)	18.00 ( $\pm$ 9.62)	16.68 ( $\pm$ 9.11)
<b>PD <math>\geq</math> 7 mm (%)</b>	0.50 ( $\pm$ 1.41)	0.76 ( $\pm$ 1.19)	0.63 ( $\pm$ 1.17)
<b>CAL <math>\leq</math> 1 mm (%)</b>	73.62 ( $\pm$ 28.44)	67.76 ( $\pm$ 29.89)	67.40 ( $\pm$ 28.52)
<b>CAL 2-4 mm (%)</b>	18.87 ( $\pm$ 15.12)	25.65 ( $\pm$ 14.68)	23.02 ( $\pm$ 14.50)
<b>CAL <math>\geq</math> 5 mm (%)</b>	7.50 ( $\pm$ 13.66)	11.61 ( $\pm$ 19.54)	9.59 ( $\pm$ 17.00)

Data are presented as mean ( $\pm$  standard deviation) or frequency. There was no significant difference between patients with active IBD and those in remission. VPI: visible plaque index; BoP: bleeding on probing; PD: probing depth; CAL: clinical attachment loss.

### **Gingival versus intestinal tissue**

Cytokine expression in gingival and intestinal tissues is depicted in Figure 1. Cytokine levels were similar in intestinal tissue between CD and UC patients (data not shown), so that CD and UC were analysed as a unique IBD group. Regarding gingival tissue, IL-23 showed increased levels in patients with CD in comparison to patients with UC ( $p=0.002$ ).

The expression of IL-23 ( $p=0.033$ ) and INF- $\gamma$  ( $p=0.023$ ) were significantly increased in gingival tissue, whereas IL-31 ( $p=0.028$ ) and TNF- $\alpha$  ( $p=0.018$ ) were

significantly increased in intestinal tissue. There was a trend towards higher levels of IL-17A ( $p=0.085$ ) and lower IL-17F in gingival tissue ( $p=0.060$ ).

### **Disease activity versus remission**

The expressions of IL-1 $\beta$ , IL-4, IL-6, IL-17A, IL17F, IL-21, IL-31, IL-33, and sCD40L were significantly increased in intestinal tissue from patients in IBD activity (Figure 2). TNF- $\alpha$  showed a trend towards increased levels in patients with active IBD ( $p=0.088$ ). In gingival tissue, IL-4, IL-10 and IL-21 exhibited significantly higher levels in patients in IBD activity (Figure 3). Also, there was a trend towards higher IL-1 $\beta$  levels in patients with active disease ( $p=0.076$ ).

### **Inflammation score**

The inflammation score comprised four cytokines, IL-1 $\beta$ , IL-6, IL-21, and sCD40L. The score was significantly higher in intestinal tissue from patients in IBD activity ( $p=0.006$ ). This score was also significantly increased in gingival tissue from patients in IBD activity ( $p=0.011$ ; Figure 4). There was a significant correlation between gingival and intestinal inflammation scores ( $\rho=0.548$ ;  $p=0.01$ ). Interestingly, no cytokine in intestinal tissue showed a significant correlation with its equivalent in gingival tissue.

### **Cytokine networks**

The cytokine networks of gingival and intestinal tissues are depicted in figure 4. Overall, the intestinal network (fig. 4B) shows an increase in the number of connections in comparison with the gingival network (fig. 4A). Interestingly, no negative correlation was observed in either of the networks. The network of the gingival tissue was characterised by the strong correlations ( $\rho>0.8$ ) between IL-17F and IL-25 and between IL-4 and IL-21. In the intestinal tissue, strong correlations were observed between IL-1 $\beta$  and TNF- $\alpha$ , TNF- $\alpha$  and IL-10, IL-10 and IL-31, IL-4 and IL-6, and between IL-6 and sCD40L. IL-23 presented no significant correlation with other cytokines in intestinal tissue.

### **Anti-TNF- $\alpha$ drug**

Four patients were taking anti-TNF- $\alpha$  agent. Therefore, an analysis restricted to those not taking anti-TNF- $\alpha$  agent (n=17) was performed in order to compare the cytokine levels in gingival and intestinal tissue. There were trends towards increased TNF- $\alpha$  levels (p=0.068) and decreased IL-17A levels (p=0.093) in the intestinal tissue when compared to gingival tissue. Regarding the comparisons between activity and remission, and the inflammation score, the results remained largely unaltered by the removal of these four patients.

## DISCUSSION

This study reported an increased inflammatory status in the gingival tissue of patients with an active IBD in comparison with patients in remission, as evidenced by significantly higher levels of IL-4, IL-10, IL-21 and higher inflammation score, and a trend towards increased IL-1 $\beta$ . To the best of our knowledge, this is the first study to show that IBD activity has an influence on gingival tissue. IBD activity increases the cytokine response not only in intestinal tissue[7], but also in sera[8, 9] and in saliva[17]. Szczeklik *et al.* have found increased salivary levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in patients with active CD[17]. Regarding gingival fluid, a previous study from our group found that IBD had a relatively small effect on the local cytokine expression in patients with untreated periodontal disease, but there was no comparison made between patients with active IBD and those in remission[6]. This study now adds evidence that IBD activity also affects gingival tissue, which merits further investigation concerning whether or not intestinal activity modulates the development of periodontal disease.

Aiming to assess the overall impact of IBD activity on gingival tissue, we created an inflammation score using intestinal cytokines exhibiting accuracy >0.8 in discriminating patients with active disease from those in remission. Afterwards, the score was applied to gingival tissue using the same cytokines and it was significantly higher in gingival tissue from patients with active IBD. Also, the score in gingival tissue correlated significantly with the one in intestinal tissue, whereas no cytokine in gingiva correlated with their counterpart in intestine. The development of inflammation or immune scores has been performed for cancer[18], rheumatoid arthritis[19], myocardial dysfunction[20], and risk of infections[21]. Analysis of biomarker combinations has also been performed in IBD. Serum IL-6 level, alone or in association with other biological markers, might be

useful for predicting the course of quiescent CD[22]. We believe that the combination of cytokines, rather than single cytokines, provide a better route to developing a useful clinical score to monitor patients.

We found increased levels of IL-23 and INF- $\gamma$ , and decreased levels of IL-31 and TNF- $\alpha$  in gingival tissue in comparison with intestinal tissue. However, the levels of the other 11 cytokines were similar in both tissues. Despite the regional specialisations of different mucosae, these results point towards the possibility that both periodontal and intestinal lesions might resemble each other in their inflammatory mechanisms[23, 24]. These findings, and the fact that IBD activity seems to increase the inflammation response in gingival tissue, might support the concept of a common mucosal immunological system, where the stimulation of one mucosal compartment can influence a distant mucosal site, and function as a global organ that affects immune development[25, 26].

Regarding the cytokine correlations, the intestinal network showed a higher number of connections than the gingival network did. TNF- $\alpha$  and IL-4 took a central position in gingival and intestinal networks, respectively. In both tissues, we observed a correlation between Th17 cytokines, such as IL-17A, IL-23, IL-22 and IL-21 in gingiva and IL-17F, IL-21 and IL-22 in intestine. In fact, Th17 cytokines play a significant role in both IBD and periodontitis[7, 10, 13]. In addition, a correlation between IL-1 $\beta$ , TNF- $\alpha$  and IL-17A was observed in both tissues, highlighting a pro-inflammatory clustering in both diseases. IL-17A and INF- $\gamma$  were correlated in intestinal tissue, a finding consistent with the pathogenic role played by Th17/Th1 cell subset in intestinal inflammation[10].

Given the significant role played by IL-23 in intestinal inflammation[27, 28], a surprising finding of our study was the low levels of IL-23 in intestinal tissue even in patients with active IBD. In addition, IL-23 did not correlate with any other cytokine in the intestinal network. However, increased IL-23 levels have been recorded in inflamed mucosa from IBD patients, as has its correlation with disease activity[9, 11, 29]. The reason for this discrepancy is unknown, but IL-23 levels could decrease with anti-inflammatory treatment[29]. Since all patients were taking drugs for IBD, it would appear that IL-23 was somehow more affected than the other cytokines.

With regards to IBD treatment, four patients were taking an anti-TNF- $\alpha$  drug. Thus, we performed one analysis restricted to patients not taking the drug. IL-23, INF- $\gamma$ , IL-31 and TNF- $\alpha$  lost significance in the comparison between gingival and intestinal tissues; we believe that this was mainly caused by the reduction in the sample size. However, it should be kept in mind that anti-TNF- $\alpha$  drugs might affect not only TNF- $\alpha$ ,

but may also affect Th1, Th17 and Th22 cells[30]. Also, supernatants from infliximab-treated colonic biopsies exhibited lower TNF- $\alpha$ , IL-17, IL-6 and IL-8 concentrations[31].

This study has limitations that should be taken into consideration when evaluating its results. As a cross-sectional study, it implies the limitations of the design itself. Furthermore, the number of subjects with active disease and in remission was small, and the findings should be confirmed in larger cohorts. As there were no significant difference in cytokine levels between CD and UC patients, we grouped them together to form one single IBD group. Granlund *et al.* have shown a similarity between the gene expressions in inflamed colonic mucosa from UC and CD patients [32]. Our study opens up a new venue in the investigation of the relationship between IBD and periodontitis.

In conclusion, the activity of IBD influenced the cytokine expression in gingival tissue from patients with periodontitis, as evidenced by increased levels of IL-4, IL-10 and IL-21, and a higher inflammation score in patients with active disease.

## FUNDING

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## FIGURE LEGENDS

**Figure 1.** Median fold change of cytokine expression between gingival and intestinal tissue from patients with periodontitis and inflammatory bowel disease, n=21.

\*Indicates a statistically significant difference.

**Figure 2.** Cytokine expression in intestinal tissue from patients with active IBD (n=8) and those in remission (n=13).

\*Indicates a statistically significant difference.

**Figure 3.** Cytokine expression in gingival tissue from patients with active IBD (n=8) and those in remission (n=13).

\*Indicates a statistically significant difference.

**Figure 4.** Comparison of inflammation score in gingival and intestinal tissues between patients with active IBD and those in remission.

\*Indicates a statistically significant difference.

**Figure 5.** Cytokines network in gingival (A) and intestinal tissue (B).

