

# Oral coinfection can stress peripheral lymphocyte to inflammatory activity in leprosy

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## ABSTRACT

**Introduction:** This study evaluated the intracellular profile of interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-10 (IL-10) and interferon- $\gamma$  (IFN- $\gamma$ ) in peripheral blood mononuclear cells (PBMCs) from leprosy patients based on oral infections presence to determine whether these coinfections could be associated with pro-inflammatory activity in leprosy. **Methods:** Leprosy patients regardless of clinical form and specific leprosy treatment (n=38) were divided into two groups: Group I – leprosy patients with oral infections (n=19), and Group II – leprosy patients without oral infections (n=19). Non-leprosy patients presenting oral infections were assigned to the control Group (n=10). Intracellular IL-2, IL-4, IL-10 and IFN- $\gamma$  production was evaluated by flow cytometry (FACS) before and 7 days after controlling the oral infection in the Group I, before and 7 days after dental prophylaxis in the Group II, and during oral infection process in control Group. **Results:** Low percentages of CD3<sup>+</sup> lymphocytes bearing IL-2, IL-10 and IFN- $\gamma$  were observed in the Group I and Group II at baseline and 7 days after therapy or prophylaxis compared to controls. Group I showed reduced percentages of IL-4 at baseline and 7 days after therapy compared to controls, or at baseline of Group II, and the Group II showed reduced percentages of CD3<sup>+</sup> cells bearing IL-4 compared to control. An increase of the percentages of CD3<sup>+</sup> cells bearing IL-4 was observed in the Group I after the oral infections treatment. **Conclusions:** The occurrence of oral infections favors the intracellular cytokines expression and, probably, the inflammatory reaction operating as a stimulatory signal triggering the leprosy reactions.

**Keywords:** Leprosy reaction. *Mycobacterium leprae*. Oral infection. Coinfection. Cytokines. Intracellular staining.

## INTRODUCTION

Leprosy reactional episodes consist of acute inflammatory episodes and are complex situations of the course of the disease. The understanding of the physiopathology of these episodes is fundamental to facilitate the treatment and control of leprosy, since these reactions can be responsible for much of the permanent nerve damage leading to disability and deformities<sup>1-3</sup>.

Cytokine measurement during the reactional episodes has been reported in many studies, especially T cell biomarkers analyzed in plasma, serum, skin lesions and saliva<sup>3-10</sup>, demonstrating their potential to play a significant role in the classification, prognosis and evolution of leprosy. We have been studying the relationship between these episodes and infectious processes such as chronic oral infections<sup>11</sup>. Since oral infections can induce over-stimulation of the host immune

system through the release of inflammatory peptides<sup>12,13</sup>, it is reasonable to consider the possibility that these infectious processes act as an inducing and exacerbating or maintaining factor of leprosy reactions. Therefore, it seems important to determine the immune-cellular behavior in leprosy patients with oral infections to investigate whether this coinfection could influence the course of leprosy disease.

In a previous study evaluating the expression of pro-inflammatory serum biomarkers we observed that leprosy patients with oral infections presented more leprosy reactions associated with higher C-reactive protein (CRP), chemokine IP-10, interleukin-1 (IL-1) and interleukin-6 (IL-6) levels than leprosy patients without oral infections, suggesting that oral infections can act as a maintenance factor of the pro-inflammatory state<sup>14,15</sup>. However, these results do not show the real participation of peripheral blood cells and the amount of cytokines produced by each cell population, compromising the understanding of the inflammatory response. Thus, the aim of this study was to determine the intracellular profile of pro-inflammatory cytokines [interleukin-2 (IL-2) and interferon- $\gamma$  (IFN- $\gamma$ )] and anti-inflammatory cytokines (IL-4 and IL-10), in peripheral blood mononuclear cells (PBMCs) from leprosy patients stratified according to the presence of chronic oral infections to determine whether these coinfections could be associated with pro-inflammatory activity in leprosy.

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## METHODS

### Patients

Forty-four leprosy patients regardless clinical form and specific leprosy treatment with multidrug therapy (MDT), as proposed by the World Health Organization (WHO), participated in the study. The diagnosis of leprosy was made based on the Ridley and Jopling classification criteria<sup>16</sup>. Patients were selected consecutively based on presence of oral infections at the Leprosy Clinics of the School of Medicine of Ribeirão Preto, University of São Paulo, and were divided into two groups matched by age, sex, and ethnic: group I consisted of leprosy patients presenting some oral infections, and group II consisted of leprosy patients without oral infections. The oral infections considered were: periodontal diseases (PD), irreversible pulpitis (IP), pulpal necrosis (PN) and inflammatory periapical lesions (IPL). Inclusion criteria were: at least one tooth with a probing pocket depth > 4mm at two sites, or at least one tooth with some symptomatic or asymptomatic dental diseases (IP, PN or IPL). The control group consisted of patients without leprosy presenting oral infections in order to evaluate if the oral infection alone could stimulate cytokines production at the same way than in leprosy patients. Subjects were excluded if they presented a co-existing local or systemic infection [human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis B virus (HBV)] or diabetes mellitus, and if they had received antimicrobial treatment for oral infections in the previous 6 months.

### Study design

After the diagnosis of leprosy and of oral disease, patients from the three Groups (I, II and control) were instructed about oral hygiene techniques and supragingival prophylaxis. Leprosy patients were reexamined 7 days after completion of the dental and/or periodontal treatment (Group I) or dental prophylaxis (Group II). Blood samples were collected into heparinized vacutainers immediately before and 7 days after controlling the oral infection in the Group I, before and 7 days after dental prophylaxis in the Group II, and during oral infection process in control group.

### Intracellular cytokine evaluation

Fluorescein isothiocyanate-conjugated (FITC) monoclonal antibodies for cell surface antigens (anti-CD3) or phycoerythrin-conjugated (PE) monoclonal antibodies to human cytokines (anti-IFN- $\gamma$ , anti-IL-2, anti-IL4 and anti-IL-10) were obtained from Becton Immunocytometry System (BD, San Jose, CA, EUA).

**Cell stimulation:** to evaluate the percentage of cluster of differentiation 3 (CD3)<sup>+</sup> cells presenting IL-2, IL-4, IL-10 and IFN- $\gamma$  staining, PBMCs were separated by gradient density, resuspended in tissue culture (RPMI, Sigma), and stimulated with 25 $\mu$ g/mL of 4 $\alpha$ -phorbol 12-myristate 13-acetate (PMA, Sigma) and 1 $\mu$ g/mL of ionomycin (IONO, Sigma) in the presence of brefeldin (BF) for 4h (IFN- $\gamma$ ) or 24 h (IL-2, IL-4 and IL-10) at 37°C with 7% CO<sub>2</sub>.

**Staining for intracellular antigens:** all incubations were performed in the dark. Cells were stained for cell surface antigens using 1 $\mu$ L of anti-CD3 FITC for 20min at 4°C. Staining was stopped and cells were fixed with 1mL fluorescence-activated cell sorting (FACS) lysing solution (BD) and permeabilized with 500 $\mu$ L of a FACS permeabilizing solution (BD) at room temperature. Next, 3 $\mu$ L of each anti-cytokine was added to each tube and the preparation was incubated at room temperature for 30 minutes. Finally, cells were resuspended in 500 $\mu$ L of phosphate-buffered saline (PBS) with 1% paraphormaldehyde (Sigma).

**Flow cytometry analyses (FACS).** Intracellular production was evaluated by FACS. Samples were run on a FACSort and CellQuest software (BD). A total of 10,000 events were obtained for CD3<sup>+</sup> samples.

### Statistical analysis

The differences in age between the three groups were analyzed by the Kruskal-Wallis test. The association between gender and the three groups, and between clinical classification, reactional episodes and the leprosy groups were analyzed by the chi-Square test. The association between race and the three groups was analyzed by Fisher's exact test. The differences in intracellular cytokines between the three groups (Group I, II, and controls) were compared by the Kruskal-Wallis test with a Dunn post-test, and the differences between baseline values were compared to those obtained after 7 days (only for groups I and II) by the Wilcoxon test. These analyses were performed using the GraphPad Prism software (San Diego, CA, USA). Data were reported as absolute and percentage frequencies, medians and range or means and standard deviations (SD), and the level of significance was set at 5% in all analyses.

### Ethical considerations

The study was approved by the Ethics Committee of Medical School of Ribeirão Preto, São Paulo University, Brazil (#15688/2005). All subjects gave written informed consent to participate.

## RESULTS

### Patients

Six of the initial 44 leprosy subjects were excluded from the study, four of them because they did not conclude the dental treatment, and two because of a worsening of their general condition. The final enrolled sample consisted of 38 leprosy patients (28 men and 10 women, mean  $\pm$  SD age 43.92  $\pm$  2.12 years; range 18-81 years) before, during or after specific leprosy treatment (multidrug therapy-WHO), and 10 healthy patients, as a control Group (**Table 1**). Group I consisted of 19 leprosy patients (13 men and 6 women, mean age 45.05  $\pm$  6.3 years; range 18-72 years) presenting some oral infections: seven patients presented dental diseases (IP, PN and IPL), 3 presented PD, and 9 presented a combination of dental diseases (PN and IPL) and PD. In this group, 8 patients presented lepromatous leprosy (LL), 4 were borderline lepromatous (BL), 2 borderline borderline (BB), 3 borderline tuberculoid (BT), and

**TABLE 1 - Demographic and clinical data of the leprosy patients presenting oral infections (Group I), leprosy patients without oral infections (Group II) and non-leprosy patients presenting oral infections (Control group).**

Variables	Group I (n=19)	Group II (n=19)	Control group (n=10)	p-value
<b>Age</b>				
mean ± SD	45.05 ± 6.3	42.8 ± 13.4	45.7 ± 8.48	0.10*
<b>Gender</b>				
male	13 (68.4)	15 (78.9%)	6 (60%)	0.54**
female	6 (31.6%)	4 (21.1%)	4 (40%)	
<b>Race</b>				
white	13 (68.4%)	16 (84.2%)	6 (60%)	0.46†
african-American	5 (26.3%)	3 (15.8%)	3 (30%)	
asian	1 (5.3%)	0 (0.0%)	1 (10%)	
<b>Clinical classification</b>				
TT	2 (10.5%)	3 (15.8%)	-	0.53**
BT	3 (15.8%)	7 (36.8%)	-	
BB	2 (10.5%)	2 (10.5%)	-	
BL	4 (21.1%)	2 (10.5%)	-	
LL	8 (42.1%)	5 (26.3%)	-	
<b>Reactional episodes</b>				
ENL	15 (78.9%)	2 (10.5%)	-	< 0.01**
RR	3 (15.8%)	4 (21.1%)	-	
none	1 (5.3%)	13 (68.4%)	-	

TT: tuberculoid leprosy; BT: borderline tuberculoid leprosy; BB: borderline; BL: borderline lepromatous leprosy; LL: lepromatous leprosy; RR: reversal reaction; ENL: erythema nodosum leprosum; SD: standard variation. \*Kruskal-Wallis test with a Dunn post-test; \*\*Chi-square test; † Fisher's exact test.

2 tuberculoid (TT). Group II consisted of 19 patients (15 men and 4 women, mean age  $42.8 \pm 13.4$  years; range 21-81 years) without oral infections. In this group, 5 patients presented LL, 2 BL, 2 BB, 8 BT and 2 TT form. The control Group consisted of 10 male patients (mean ± SD age  $45.7 \pm 8.48$  years; range 42-58) presenting oral infections: three patients with dental diseases (IP and PN), 3 with PD, and 4 with a combination of dental diseases (IP) and PD.

#### Reactional episodes

At the time of recruitment, high number of patients from Group I presented RE, compared with Group II ( $p < 0.01$ , chi-Square test; **Table 1**): 15/19 (78.9%) patients in group I presented ENL (8 LL, 4 BL, 2 BB, and 1 BT), three (15.8%) patients had reversal reaction (2 BT and 1 BL patients) and only one (5.3%) patient did not present reactional episodes. Two (10.5%) of nineteen patients in Group II presented ENL (1 LL and 1 BL patients), four (21%) patients had reversal reaction (2 TT, 1 BT and 1 BB patients) and thirteen (68.4%) patients did not present reactional episodes. After dental and/or periodontal treatment, 68.4% (13/19) of Group I patients presented improvement of the

clinical manifestations of reactional episodes, mainly erythema nodosum leprosum (ENL). No clinical alteration was verified in Group II between the two analyses.

#### Intracellular cytokines

Low percentages of CD3+ lymphocytes bearing IL-2, IL-10 and IFN- $\gamma$  were observed in the Group I (leprosy patients with oral infections) and Group II (leprosy patients without oral infections) at baseline and 7 days after therapy or prophylaxis compared to controls (non-leprosy patients with oral infection) (**Table 2** and **Figures 1A, 1C** and **1D**). Likewise, group I showed reduced percentages of IL-4 at baseline and 7 days after therapy compared to controls ( $p < 0.05$ , Kruskal-Wallis test with a Dunn post-test), and the Group II showed reduced percentages of CD3+ cells bearing IL-4 compared to control group (**Table 2** and **Figure 1B**), although there was no statistical difference. An increase of the percentages of CD3+ cells bearing IL-4 and IFN- $\gamma$  was observed in the Group I after the oral infections treatment ( $p < 0.05$ , Wilcoxon test) (**Table 2** and **Figures 1B** and **1D**). There was no evidence of differences in the remaining comparison between groups.

TABLE 2 - Percentage of CD3<sup>+</sup> T cells producing IL-2, IL-4, IL-10, and IFN- $\gamma$  after in vitro activation with PMA and ionomycin from PBMCs of leprosy patients with oral infections (Group I), leprosy patients without oral infections (Group II) and non-leprosy patients with oral infections (control Group). Results are expressed as median (range).

	Group I (n=19)		Group II (n=19)		Control Group (n=10)
	baseline	7 days after therapy	baseline	7 days after prophylaxis	
CD3 <sup>+</sup> /IL-2	17.19 (0.00 – 43.8)	23.57 (0.00 – 52.48)	21.24 (0.02 – 51.5)	20.20 (0.00 – 50.8)	43.08 (28.86 – 84.42)
CD3 <sup>+</sup> /IL-4	0.14 (0.00 – 1.61)	0.61 (0.00 – 5.91)	0.64 (0.00 – 2.74)	1.12 (0.00 – 4.12)	1.89 (0.00 – 4.17)
CD3 <sup>+</sup> /IL-10	0.00 (0.00 – 4.58)	0.61 (0.00 – 1.12)	0.00 (0.00 – 0.57)	0.00 (0.00 – 0.47)	0.41 (0.00 – 4.71)
CD3 <sup>+</sup> /IFN- $\gamma$	12.07 (0.00 – 31.68)	13.96 (2.62 – 36.7)	11.31 (31.22 – 0.04)	12.10 (30.2 – 1.68)	28.86 (16.08 – 58.14)

CD3<sup>+</sup>: cluster of differentiation 3<sup>+</sup>; IL-2: interleukin-2; IL-4: interleukin-4; IL-10: interleukin-10; IFN- $\gamma$ : interferon- $\gamma$ ; PMA: 4 $\alpha$ -phorbol 12-myristate 13-acetate; PBMCs: peripheral blood mononuclear cells.

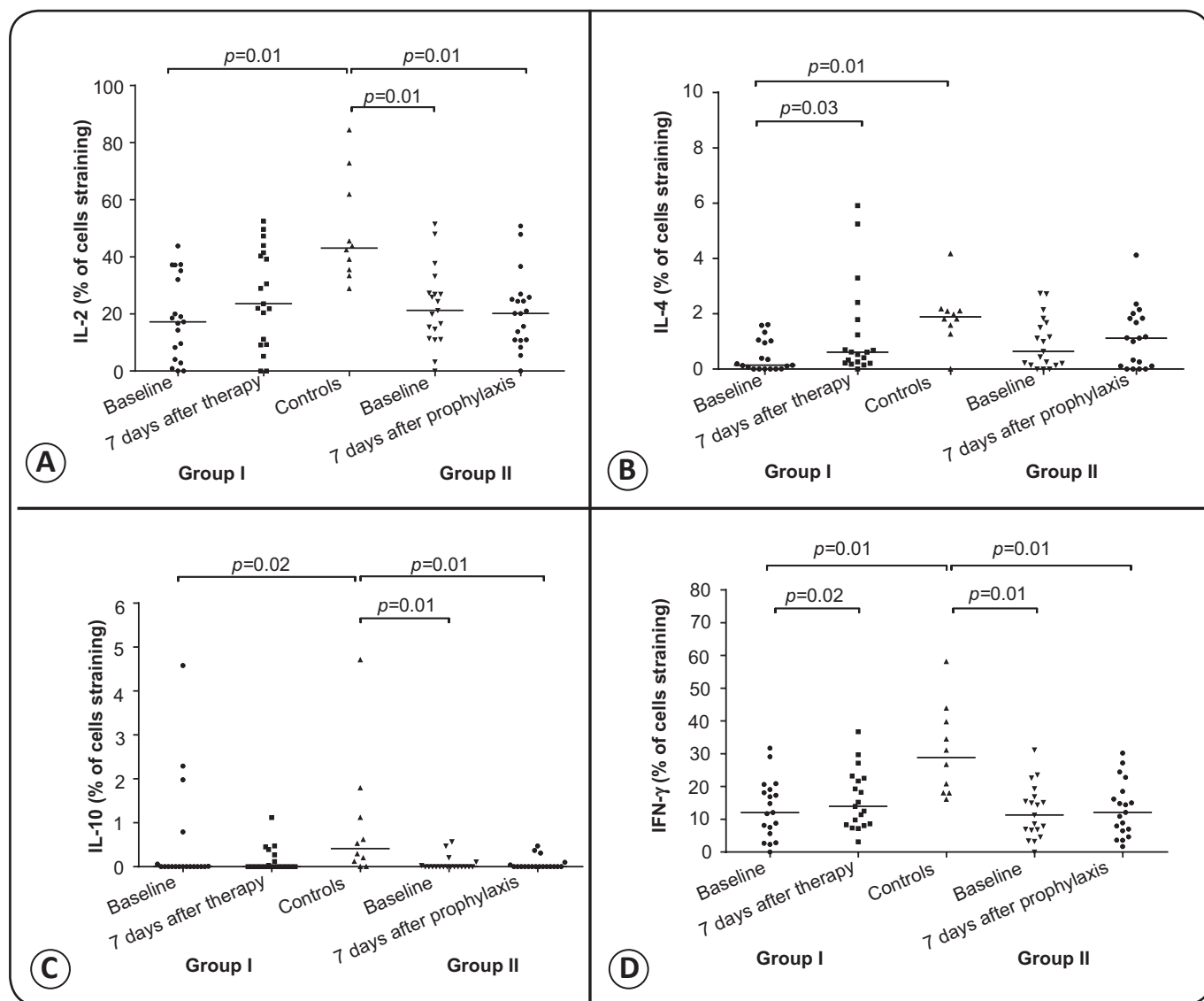


FIGURE 1 - Comparisons between the percentages of CD3<sup>+</sup> T cells producing IL-2 (A), IL-4 (B), IL-10 (C) and IFN- $\gamma$  (D) from PBMCs of leprosy patients with oral infection (group I; n=19), at baseline and 7 after oral infections therapy; leprosy patients without oral infection (group II; n=19), at baseline and 7 days after dental prophylaxis; and non-leprosy patients with oral infection (control; n=10), during the infectious process. The horizontal lines represent the median of each group. PBMCs were stimulated with PMA and ionomycin for 4h (IFN- $\gamma$ ) or 24h (IL-2, IL-4 and IL-10). The horizontal lines represent the median of each group.

IL-2: interleukin-2; IL-4: interleukin-4; IL-10: interleukin-10; IFN- $\gamma$ : interferon- $\gamma$ ; PBMCs: peripheral blood mononuclear cells. PMA: 4 $\alpha$ -phorbol 12-myristate 13-acetate.

## DISCUSSION

Usually the peripheral profile of cytokines in leprosy (serum and plasma) reflects the measure of many sources of cytokine production<sup>17,18</sup> without identifying which specific cell is really compromised<sup>19,20</sup>. It has been observed that PBMCs present different levels of proliferation index of cytokines when they are stimulated *in vitro* with *Mycobacterium leprae* extracts<sup>21-23</sup>. On the other hand, we observed that the leprosy reactional episodes are frequent in patients presenting oral infections<sup>14,15</sup> and other coinfections<sup>24</sup>. Therefore, this study attempted to clarify whether chronic oral infections could influence the intracellular production of cytokines in leprosy patients, and to verify the relationship with the development of reactional episodes.

Cytokines like IFN- $\gamma$  are believed to be essential for the activation of macrophages and for inducing the destruction of intracellular *Mycobacterium leprae*, leading to specific activation and differentiation of T cells, modulated by IL-2, IL-12 and IL-18, resulting in infection control and granuloma formation<sup>21,26,27</sup>. At the other end of the leprosy spectrum, the immunity is mediated by IL-4 and IL-10, regulatory cytokines able to block macrophage activation and exacerbation of humoral immunity, resulting in an increased bacterial load<sup>28</sup>.

The evaluation of intracellular cytokines that have a pathogenic role in leprosy showed a low quantity of CD3<sup>+</sup> cells that stained for IL-2 in leprosy patients, more accentuated in patients with oral coinfection compared to control, suggesting that the infectious process by *M. leprae* can reduce the IL-2 response. Similar results were found in CD3<sup>+</sup> cells that stained for IFN- $\gamma$  with significantly diminished detection in leprosy patients compared to controls, and the most interesting find was that oral infections treatment can induce an increase of IFN- $\gamma$  (**Figure 1D**). Then, it we may suggest that the differentiation of CD3<sup>+</sup> cells to the Th1 type is depressed in leprosy and the oral coinfection could act by augmenting the pro-inflammatory response mediated by IFN- $\gamma$ .

CD3<sup>+</sup> cells stained for IL-4 showed low percentages in leprosy patients with oral infections compared to leprosy patients without oral infection and controls, and the oral infection treatment induced an increased production of this cytokine ( $P=0.03$ ; **Figure 1B**), suggesting that oral infection can depress the immunoregulatory activity of IL4 while maintaining the pro-inflammatory activity of IFN- $\gamma$ , resulting in exacerbation of the inflammatory reaction. Additionally, the percentage of CD3<sup>+</sup> cells stained for IL-10 was similar in leprosy patients with oral infection (Group I) and leprosy patients without oral infection (Group II), and the oral infection control did not influence these results. Both leprosy groups (I and II) showed reduced production of this cytokine compared to control group. Since IL-10 has been considered to be an anti-inflammatory cytokine that can regulate the Th1 pattern of the cellular immune response<sup>29,30</sup>, it is possible to consider that the coinfection acts on the course of leprosy infection in two ways: worsening the specific immunological response of the leprosy patients and favoring the inflammatory reaction, clinically manifested as a reactional episode.

The coexistence of oral chronic infections with leprosy can modulate the inflammatory reaction by elevation of the intracellular inflammatory markers expression, probably stimulating a spill-over of these inflammatory products into the peripheral circulation where they can act as inducers of an inflammatory reaction, exacerbating the insidious chronic evolution of leprosy and, consequently, acting like an inducer, stimulatory or maintaining factor in leprosy reactions<sup>14,15,24</sup>. In addition, the presence of these chronic infections can influence the production of intracellular cytokines, and can reflect the fact that most of the leprosy patients with coinfection (Group I) presented reactional episodes, corroborating the hypothesis of a synergistic action of the two kinds of inflammatory reaction on the course of leprosy. A limitation of the study was not match properly the leprosy groups by clinical form, and the small number of patients. Possibly, studies with larger numbers of patients properly matched by clinical leprosy forms might assess the impact of oral coinfections on occurrence of leprosy reactional episodes.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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