

Association between COX-2 rs 6681231 Genotype and Interleukin-6 in Periodontal Connective Tissue. A Pilot Study

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Abstract

Objectives: The aim of this pilot study was to investigate associations between IL-6 and COX-2 expression in gingival biopsies and both clinical diagnosis and genotypes in the IL-6 and COX-2 genes.

Design: A case-control study included 41 gingival biopsies obtained from Caucasian patients grouped according to clinical diagnosis of gingival health (n = 10), gingivitis (n = 15) or chronic periodontitis (n = 16). Immunohistochemistry analyses were performed to determine COX-2 expression in lamina propria, IL-6 expression in lamina propria and gingival epithelium and level of inflammatory cell infiltrate. Individual DNA was extracted and genotyped by real-time PCR for *IL6* SNPs rs 2069827 and rs 2069825 and for *COX-2* rs 6681231.

Results: The percentage of cellular COX-2 expression was associated with the extent of periodontal disease (Arbes index $p = 0.026$) and inflammatory infiltrate ($p < 0.0001$). No association was observed between *IL6* haplotypes and cells positive to IL-6 or COX-2 in gingival tissues. The *COX-2* rs 6681231 was associated with cells positive to IL-6 in the connective tissue ($p = 0.032$).

Conclusions: COX-2 expression in gingival tissues may be a marker of periodontal disease severity. COX-2 rs 6681231 may be associated with IL-6 local production in gingival tissues.

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Introduction

The systemic immune response, genetic factors and environmental factors affect the risk of developing periodontitis [1,2]. In recent years, studies have demonstrated that elevated levels of a variety of inflammatory biomarkers [3,4] and genetic variants of some cytokines confer susceptibility to periodontitis [5–7].

Interleukin-6 (IL-6) is a cytokine produced by lymphocytes, monocytes, fibroblasts and endothelial cells, with functions in the systemic inflammatory response and on regulation of the acute phase response [8]. In particular, IL-6 is able to stimulate the synthesis of all the acute phase proteins involved in the inflammatory response C-reactive protein, serum amyloid A, fibrinogen, α 1-chymotrypsin and haptoglobin [9]. Cyclooxygenase-2 (COX-2) is an enzyme involved in the conversion of arachidonic acid to prostaglandins, with a consequent important role in inflammatory responses, also in the periodontium [10,11].

COX-2 expression is induced by cytokines [12] and, on the other hand, prostaglandins are important regulators of IL-6 production by gingival fibroblasts [13]. Therefore, within the multitude of actions on a wide range of cells and matrix structures in complex organ systems, IL-6 and COX-2 have the potential to influence one another.

A large number of scientific papers investigated the role of gene variants (polymorphisms) in host responses in periodontitis, and in the progression of the disease [14]. *IL6* polymorphisms and haplotypes have been shown to affect IL-6 transcription and therefore circulating interleukin-6 levels [15–17]. A recent meta-analysis of the *IL6* –174 polymorphisms did not show any association for this polymorphism with chronic periodontitis [14]. However, an association was found between IL-6 –174 and aggressive periodontitis in a separate meta-analysis [18]. Similarly, genetic regulation of COX-2 production has been associated with

periodontitis [19]. Recently, a large study in Caucasians showed an association between *COX-2* rs 6681231 genetic polymorphism and presence of aggressive periodontitis [20].

The objective of this pilot study was to investigate the association between IL-6 and COX-2 expression in gingival biopsies and both clinical presentation (disease severity) and genetic polymorphisms in the *IL6* and *COX-2* genes.

Materials and Methods

Ethical considerations

All participants signed informed consent, and the study was conducted in accordance with the Helsinki declaration (version 2002) and approved by the ethics/research committee of the University of Granada (ref 04/072011).

Clinical examination and subject selection

A case-control and analytic study was conducted in 41 adults requiring tooth extraction for caries, tooth fracture, endodontic failure or tooth mobility (>1 mm in buccal-lingual direction and cause of discomfort). Inclusion criteria were the same for all three groups: Caucasian ethnicity, age >18 years and the presence of at least 6 teeth. Exclusion criteria were periodontal treatment in the previous year, antibiotics or anti-inflammatory treatment in the previous 2 months and self-reported diagnosis of diabetes. Demographic (age, gender) and clinical data were collected by a calibrated examiner (R.M.). Gingival inflammation was assessed using the gingival bleeding index [21] and probing depth and loss of clinical attachment were determined using a PCPUNC15 periodontal probe (1 mm increments) (University of North Carolina, Hu-Friedy, Chicago, IL), at six sites per tooth (mesiovestibular, vestibular, distovestibular, mesiolingual, lingual, and distolingual). CP extent was classified by the percentage of sites with a loss of attachment ≥ 3 mm [22]. Following periodontal examination, participants were grouped according to clinical diagnosis of chronic periodontitis (CP), gingivitis (G), or gingival health (control = C). For inclusion to this study:

- Chronic Periodontitis (CP) was classified by the presence of at least 1 site with clinical attachment level (CAL) ≥ 3 mm and at least 1 site with probing pocket depth (PPD) of ≥ 6 mm
- Gingivitis (G) was defined by any gingival bleeding on probing (>0%) [21] and absence of PPD ≥ 3 mm
- Gingival health (C) was defined as no gingival bleeding on probing [21] and absence of PPD ≥ 3 mm.

Each patient contributed with one site to the study sampling procedures. All patients were treated with tooth extraction (and periodontal treatment if appropriate) at the School of Dentistry, University of Granada (Spain) from September 2010 to June 2011. Immediately before the tooth extraction, the same clinician (F.M.) used a scalpel to take a single biopsy from the mesial or distal gingival papilla of each patient after anesthetization with 2% mepivacaine. In CP patients, biopsies were obtained before tooth extraction at sites with probing depth of ≥ 6 mm with bleeding on probing and evidence of bone loss on periapical X-ray. In gingivitis patients, biopsies were obtained before tooth extraction at sites with bleeding on probing and PPD <3 mm.

Examiner's reproducibility

The examiner (author R.M.) was trained and calibrated on 10 patients probed twice before conducting periodontal examinations. Intra-examiner reliability was assessed by using the kappa statistic, which was equal to 0.78, evidencing a high degree of consistency in the observations. The examiner was also calibrated against a 'gold-standard examiner' (author F.M.) showing good concordance, with a kappa statistics of 0.82.

Immunohistochemistry analyses

- COX-2 expression: Paraffin-embedded sections were de-waxed, hydrated, and heat-treated in 1 mM EDTA buffer pH 8 for antigenic unmasking in a PT module (Thermo Fisher Scientific Inc., Waltham, MA) at 95°C during 20 min. Sections were incubated for 30 min at room temperature with anti-COX-2 (clone SP21) rabbit monoclonal antibody diluted 1:50 (Master Diagnóstica, Granada, Spain) in order to identify cell inflammatory expression. The immunohistochemistry study was done on an automatic immunostainer (Autostainer480, Thermo Fisher Scientific Inc) using the polymer-peroxidase-based method, followed by development with diaminobenzidine (Master Diagnóstica). Appropriate positive controls, as well as non-immune serum for negative controls, were run concurrently. Nuclear counterstaining was done using hematoxylin (Master Diagnóstica). The immunohistochemistry results were calculated as percentage of cells positive to COX-2 and semi-quantitatively using a 0 to 3 scale (0, absence; 1, mild [<10% of cells positives]; 2, moderate [10 to 25%]; 3, severe [>25%]).
- IL-6 expression: Five-micrometre thick sections were exposed to immunohistochemistry staining using the polyclonal

Table 1. Comparison between groups of studied periodontal, immunohistochemistry and genetic variables (n = 41).

Variables	Control (n = 10)	Gingivitis (n = 15)	Periodontitis (n = 16)	p-value
Age (years)	35.2 ± 14.7	46.6 ± 17.9	44.9 ± 9.4	0.112 ^b
Gender (male)	7 (77.8%)	6 (50.0%)	11 (68.8%)	0.469 ^a
Bleeding on probing %*	0	10.2 ± 6.9	60.6 ± 25.2	<0.001 ^c
IL-6 H+	5 (55.6%)	5 (45.5%)	4 (40.0%)	0.790 ^a
IL-6 H-	4 (44.4%)	6 (54.5%)	6 (60.0%)	
COX-2 genotype GC	1 (12.5%)	5 (38.5%)	6 (46.2%)	0.280 ^a
COX-2 genotype CC	7 (87.8%)	8 (61.5%)	7 (53.8%)	

Continuous values are expressed as mean ± standard deviation;

a: Chi square test;

b: Kruskal-Wallis test;

c: Mann-Whitney U-test. Please note: Genotype results are available in 30/41 subjects for IL6 genotypes and in 34/41 subjects for COX-2 genotype.

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Table 2. Medians and inter-quartile range of clinical (Arbes index) and histological results for subjects divided by clinical diagnosis.

Variable	Control (n = 10)	Gingivitis (n = 15)	Periodontitis (n = 16)	Comparisons between groups
Arbes index	–	–	18 (12–46)	–
Inflammatory cells detected	1 (10%)	15 (100%)	16 (100%)	<0.001 ^a
Cox2 Plasma cells	0 (0–1)	12.5 (2.5–62.5)	40 (15–100)	0.009 ^a
Cox2 Monocytes	1 (0–2)	10 (5–62.5)	40 (15–100)	0.009 ^a
IL-6 connective tissue	12.25 (11–15)	10.75 (9–12.5)	13 (10–15)	0.346 ^b
IL-6 epithelium	12.25 (7–18)	15.25 (14–20.5)	15 (9–20.5)	0.582 ^b

a: Chi square test;

b: Kruskal-Wallis test.

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antibody against human IL-6 (sc-130326), Santa Cruz Biotechnology Inc., Germany) as described before [23]. The histological measurements were performed using a microscope (Olympus BX50, Best Scientific LTD, Wroughton, UK) equipped with an imaging system (Image Pro-PLUS 4.5, Media Cybernetics, Bethesda, USA). Each slide was categorised into two zones: infiltrated connective tissue (ICT) and periphery of the ICT (non-ICT). A 400-point lattice was superimposed over the tissue area at a magnification of $\times 400$, and the number of cross-points on positive cells was counted in duplicate and expressed as a percentage of the tissue area related to the total number of points [23].

Histological evaluation of the inflammatory infiltrate

The presence of inflammatory cells (namely lymphocytes, granulocytes, monocytes/macrophages and plasma cells) in the specimens was detected microscopically and recorded. The inflammatory infiltrate level was calculated microscopically in a semi-quantitatively manner on a 4-point scale: 0, absence of any such inflammatory cells; 1, mild [$<10\%$ of lamina propria involved]; 2, moderate [10 to 25%]; 3, severe [$>25\%$].

Genetic analyses

DNA was extracted from histological sections with a commercially-available kit ('Recover All Total Nucleic Acid Isolation', Life Technologies, Paisley, UK) and genotyped for two polymorphisms in the *IL6* gene promoter region using the Applied Biosystems 7300 Real Time PCR System as previously described [24]. The studied polymorphisms were rs 2069827 and rs 2069825 in the *IL6*

gene and rs 6681231 in the *COX-2* gene. Homozygous subjects for rs 2069827 G allele and rs 2069825 C allele (no deletion) were defined as H+, while all other subjects were H- [25]. Duplicates were added to each plate to test for error rates. Each clinical sample was anonymously coded, so the immunohistochemistry and genetic analyses were performed independently and blind to clinical diagnosis.

Statistical analysis

A specific computer program (SPSS-Windows 20.0 program SPSS IBM Inc, Chicago, IL) was used for the statistical analyses. Data on IL-6 and COX-2 expression in gingival tissues were plotted and their normal distribution was confirmed. After descriptive analysis, non-parametric tests (Mann-Whitney U-test, Kruskal Wallis) and Spearman's Rho coefficient were used to evaluate the associations between IL-6 and COX-2 expression in gingival tissues (primary outcomes) and clinical diagnosis and IL-6 and COX-2 SNPs (explanatory variables). No formal sample size calculation was performed due to the pilot nature of the study. The potential effect of confounders such as gender and age was explored. The α value was set at 0.05. The tests used are reported in table footnotes.

Results

The study included 41 gingival biopsies obtained from 16 Caucasian patients with CP, 15 with G and 10 with gingival health. Table 1 shows inter-group comparisons of demographic and clinical variables (bleeding on probing) and genetic variables (*IL6* and *COX-2*). Table 2 shows histological results in patients

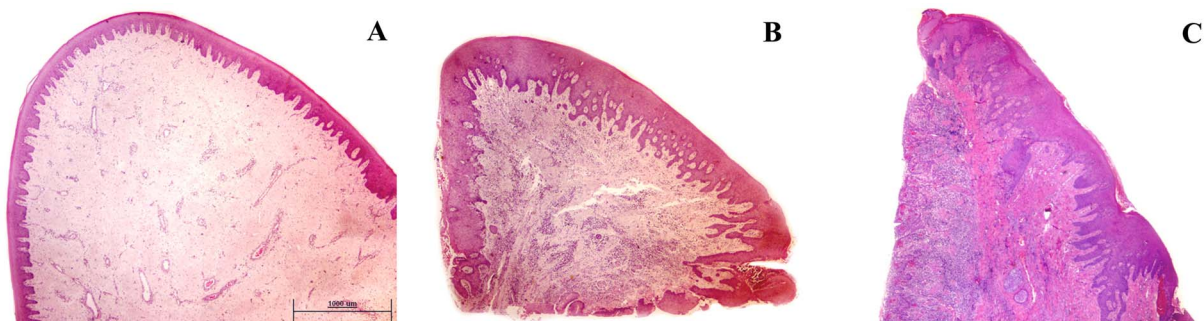


Figure 1. Panoramic microphotograph of gingival biopsies. A) Gingival health group; B) Gingivitis; C) Chronic periodontitis. (Hematoxylin-eosin, original magnification $\times 2$). (Bar 1000 μm).

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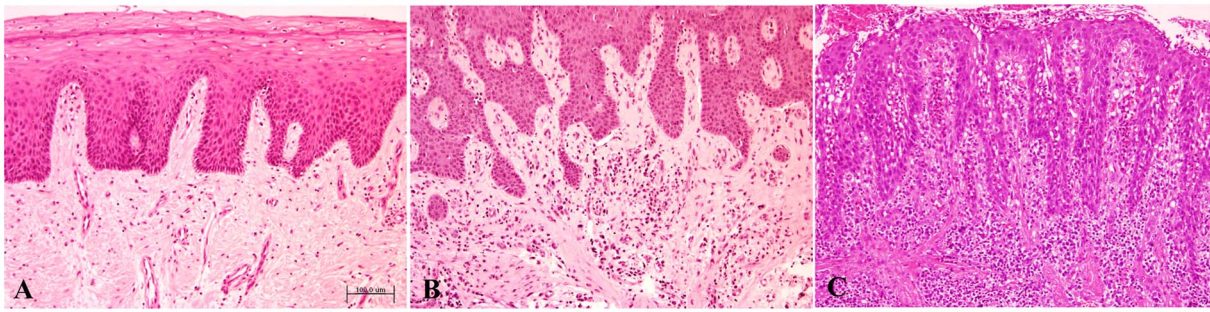


Figure 2. Gingival epithelium and lamina propria with variable chronic inflammatory infiltrate. A) Mild infiltrate in gingival health group; B) mild/moderate infiltrate in gingivitis; and C) severe infiltrate in sulcular epithelium in chronic periodontitis (Hematoxylin-eosin, original magnification $\times 10$). (Bar 100 μm).
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divided by clinical diagnosis: COX-2 immunohistochemistry expression in lamina propria (positive plasma cells and monocytes) and IL-6 expression in lamina propria and gingival epithelium. The mean \pm S.D. of percentage of sites with a loss of attachment ≥ 3 mm (Arbes index) in the CP group was 24.8 ± 18.0 . The mean probing pocket depth at the sites where the biopsy specimens were taken in CP patients was 7.12 ± 2.65 mm.

Immunohistochemistry outcomes

The clinical diagnosis was confirmed histopathologically, since inflammatory cells in the specimens were detected in only 1 out of 10 healthy, 15/15 G and 16/16 CP cases (see figures 1 and 2). The mean percentage of inflammatory cells in CP cases was lymphocytes 85% (50% of which were mature plasma cells), granulocytes 9%, and monocytes/macrophages 6% (data not presented). Figure 3 and 4 show immunohistochemistry expression of COX-2 and IL-6 respectively in the different study groups. The number of IL-6 positive cells by immunohistochemistry was similar across clinical diagnoses (CP, G, C). On the other hand, COX-2 expression in plasma cells and monocytes was associated with clinical diagnosis (both $p = 0.009$), with a gradient increase from C to G and to CP (Table 2). The percentage of cellular COX-2 expression was associated with disease severity measured as Arbes index ($p = 0.026$) and inflammatory infiltrate level (rho coefficient = 0.674, $p < 0.0001$). A positive association was also detected between inflammatory infiltrate severity and immunohistochemistry expression of COX-2 in plasma cells and monocytes of gingival lamina propria (Rho = 0.569, $p < 0.0001$; Rho = 0.565, $p < 0.0001$, respectively). No associations were

detected between immunohistochemistry outcomes and demographic factors (age and gender).

Genetic outcomes

No error rates were detected in the genetic analyses. However, some of samples yielded a small concentration of DNA, which was not suitable for genetic analysis and was not scored (15/82 IL-6 SNPs and 7/41 COX-2 SNP, overall 17.9%). The Hardy-Weinberg equilibrium was satisfied for all three studied SNPs.

The COX-2 rs 6681231 did not show any correlation with COX-2 expression at immunohistochemistry ($p = 0.884$) but was associated with cells positive to IL-6 in the connective tissue (Rho = 0.449, $p = 0.032$) (Table 3).

The IL6 haplotype was not associated with the number of IL-6 positive cells at immunohistochemistry (lamina propria $p = 0.224$, epithelium $p = 0.580$, Mann-Whitney U-test) or plasma cells and monocytes COX-2 expression ($p = 0.307$, $p = 0.134$, $p = 0.191$, respectively). IL-6 connective and epithelium immunohistochemistry expression did not correlate with COX-2 expression total percentage (Rho = 0.01, $p = 0.944$; Rho = 0.01, $p = 0.946$), plasma cells (Rho = -0.06, $p = 0.743$; Rho = -0.01, $p = 0.940$) and monocytes (Rho = 0.009, $p = 0.962$; Rho = -0.02, $p = 0.904$).

Discussion

This study showed a relationship between immunohistochemistry expression of COX-2 and periodontal pathology (gingivitis and chronic periodontitis). Furthermore, it produced initial evidence for a possible association between COX-2 genotype rs 6681231 and local IL-6 expression (measured as number of

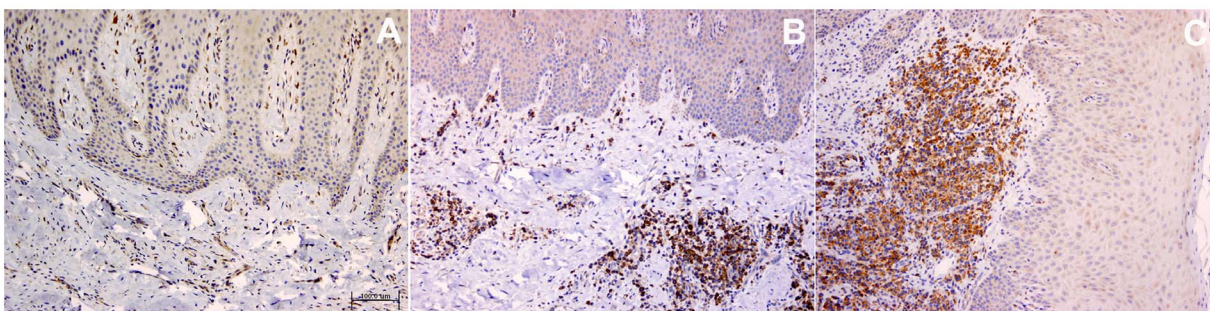


Figure 3. Representative microphotograph of COX-2 immunohistochemical expression (brown-stained) in gingival biopsies. A) Gingival health group; B) Gingivitis; C) Chronic periodontitis (Polymer-peroxidase-based method, original magnification $\times 10$).
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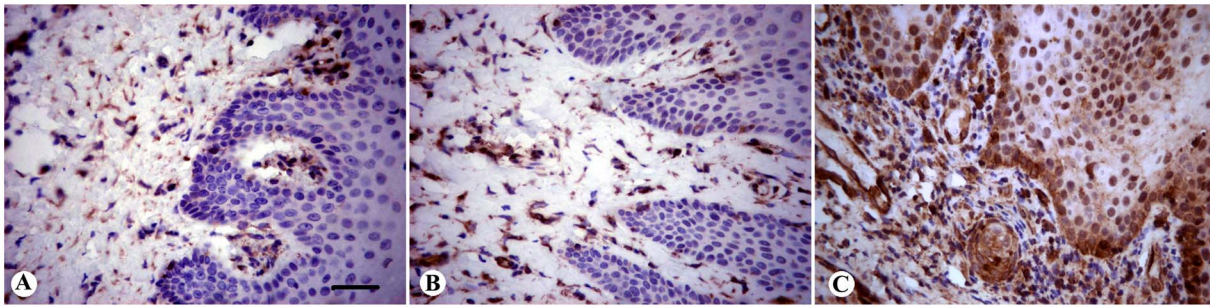


Figure 4. Representative microphotograph of IL-6 immunohistochemical expression (brown-stained) in gingival biopsies. A) Gingival health group; B) Gingivitis; C) Chronic periodontitis (original magnification $\times 40$) (Bar 100 μm).
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periodontal connective tissue cells positive to IL-6 at immunohistochemistry).

There is strong evidence that genetic, as well as environmental factors, affect the onset and severity of periodontitis [1]. Despite the publication of several genetic association studies in the field of periodontitis, including recent GWAS (genome-wide association studies), there is considerable uncertainty as to which genetic variants predispose to periodontitis. *IL6* gene promoter and *COX-2* polymorphisms are some of a few promising genetic variants studied in association with periodontitis in different populations [20,26,27,28]. However, studies for independent validation of these findings and functional studies showing the effect of these genetic variants in inducing periodontal pathology are still lacking.

To the best of our knowledge, this is the first study conducted in a Caucasian population investigating the possible effect of *COX-2* and *IL6* haplotypes on local expression of *COX-2* and IL-6 in the gingiva. Interestingly, all three studied patient groups (gingival health, gingivitis and chronic periodontitis) exhibited a similar average of IL-6 positive cells in the gingival biopsies, while *COX-2* expression was significantly associated with periodontal disease severity. The association between *COX-2* periodontal expression and periodontal pathology confirms a previous report from our group [29] and others [30], highlighting the role of this enzyme in periodontal destruction. However, it is not clear whether increased production of *COX-2* may predispose to periodontitis or if, on the other hand, *COX-2* increased expression is just a result of chronic exposure to periodontopathogenic bacteria.

In the present study, the rare allele frequency of *COX-2* polymorphism rs 6681231 (17.6%) was almost identical to the frequency reported in the HapMap CEPH Utah reference

population of European ancestry (17.8%) (www.hapmap.org). The rare G allele, which was enriched in chronic periodontitis patients in the present study, was found to be associated with aggressive periodontitis in a large North-European Caucasian population [20]. This SNP was not associated with *COX-2* expression in our sample, but showed an association with IL-6 cellular infiltrate in the connective tissue. Studies in Taiwanese and Chinese populations [19,31] reported that the -765 C polymorphism of the *COX-2* gene is associated with a decreased risk for periodontitis in these populations, especially in aggressive periodontitis. In contrast with this, functional analyses on *COX-2 -765 polymorphism have reported that the C allele may reduce the *COX-2* gene expression and consequently inhibit inflammatory responses [32,33]. A possible reason for the discrepancy is that the clinical role of *COX-2* in inflammation may be dual by initiating the process of inflammation and then later aiding in its resolution. The effects of *COX-2* genotypes in the inflammatory response through variations in *COX-2* production have been shown with relation to C-reactive protein [32] and IL-6 [34]. In particular, PEG2 is important in regulating IL-6 production and *COX-2* inhibitors have been shown to be useful in controlling fibroblast IL-6 production. The effect of *COX-2* is particularly important in gingival fibroblast IL-6 production [13], which would explain the association observed in our study with IL-6 positive cells in the connective tissue but not in the epithelial infiltrate. The lack of association between *COX-2* genetic polymorphisms and *COX-2* gingival expression may be due to the pilot nature of this study, which might help in a sample size calculation for a future study investigating this outcome.*

In conclusion, this study confirms a possible role for *COX-2* in periodontal pathology and it is the first study to provide initial evidence that the *COX-2* rs 6681231 genotype may affect IL-6 local production in gingival tissues. Limitations of this study are inherent to its pilot nature and include the small sample size, with the risk of type II error and the residual potential confounding effect of smoking. Therefore, the present findings need to be confirmed by larger investigations. If these results were confirmed, IL-6 may be considered an important mediator in the role of *COX-2* polymorphisms in periodontal pathology.

Acknowledgments

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Table 3. COX-2 gingival expression (median and inter-quartile range) and IL-6 cells positive in the periodontal connective tissue (CT) and outside CT in subjects divided by COX-2 rs 6681231 genotypes.

		Immunohistochemistry results		
		COX %	IL-6 in CT	IL-6 outside CT
COX-2 rs 6681231 genotypes	CC (n=22)	10 (5–50)	13.5 (11–16.5)*	13 (6.5–20.5)
	GC (n=12)	10 (1–50)	10 (6.5–13)*	16.7 (13–25)

*P = 0.032 Mann-Whitney U test.

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Author Contributions

Conceived and designed the experiments: FM FO MR F. Cappello ND F. Carini RM LN. Performed the experiments: FM FO MP NC RM LN.

Analyzed the data: FM FO MR LN. Contributed reagents/materials/analysis tools: FM FO F. Cappello ND MP NC LN. Wrote the paper: FM FO MR ND RM LN.

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