Positive correlational shift between crevicular antimicrobial peptide LL-37, pain and periodontal status following non-surgical periodontal therapy

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Abstract

Background. Periodontal disease represents a public health concern due to its high prevalence and uncertain recurrence after conventional treatment. Therapy outcome may be variable and given its multifactorial etiology, the precise mechanisms behind periodontitis are yet to be unveiled. In this regard, the pro-inflammatory cytokine profile has been well characterized but little is known about the anti-inflammatory cytokine and antimicrobial peptide overview prior to and after non-surgical treatment.

Methods. Sixty individuals were recruited from our University Clinic and allocated in two even groups of healthy and periodontitis subjects. A full periodontal examination was performed, and gingival crevicular fluid samples obtained at baseline and again, 4-6 weeks following scale and root planing (SRP) for the periodontitis group. Then, analyzed by ELISA kits to quantify LL-37 and interleukins 4, 6 and 10. Sex influence and the association of age and oral hygiene habits to periodontitis and treatment outcome including gingival clinical parameters and self-perceived pain were also analyzed.

Results. Higher crevicular volume and protein concentration corresponded to patients with more severe periodontitis and decreased following SRP. A positive correlational shift was also observed for LL-37 (and IL-6), self-perceived pain, and periodontal status. IL-4 and IL-10 were decreased in periodontal disease to healthy state but barely affected by conventional therapy. Levels of all mediators were irrespective of sex but ageing and tooth brushing frequency were confirmed as potential risk factors.

Conclusions. Crevicular LL-37 could stand as a reliable biomarker of both periodontal disease and the associated pain to dental probing. Also, for the prognosis following SRP therapy.

Trial registration. The study was registered in clinical trials.gov, with number NCT04404335, dated 27/05/2020.

1. Background

Periodontal disease is a multifactorial condition of inflammatory origin that results from a dysbiosis between the host immunological response and oral biofilm homeostasis [1]. In its acute form it is termed as gingivitis and may resolve without major issues within days. However, if the inflammatory situation perseveres, it may lead to degeneration of the periodontal soft tissues that support the teeth, loss of attachment and possible subsequent alveolar bone and tooth loss [2]. This manifestation is typically known as periodontitis and accounts for an estimated prevalence of 14% of all adult population according to the WHO [3].

Under healthy-state conditions, the highly vascularized periodontal tissue constitutively delivers into the gingival sulcus nutrients, serum proteins and other macromolecules such as homeostatic defensins and chemokines responsible for immune surveillance and avoidance of plaque biofilm overgrowth [4]. However, perturbations on this well-controlled niche environment may trigger an increased directional migration of neutrophils into the forming gingival pocket and activation of resident lymphocytes and macrophages. In turn, this results in the exudate of a gingival crevicular fluid (GCF) containing a plethora of inflammatory mediators including antimicrobial peptides and cytokines [5].

Among these antimicrobial peptides, cathelicidin LL-37 has drawn special attention over the past few years for being unveiled as a pivotal player in conditions that course with inflammatory-evoked tissue degeneration, particularly in the digestive and respiratory systems [6, 7]. Previous evidence suggest that neutrophils accumulate a precursor inactive form (hCAP-18) within secretory granules and, upon cell activation during the inflammatory process, LL-37 is released following enzymatic cleavage [8]. In this regard, LL-37 would exert chemoattractant and immunomodulating activities toward other immune cells [9], although interpretations on its meaning remain uncertain. Some authors point at deficiency in salivary LL-37 as a likely reason for periodontitis [10, 11], whereas others have suggested that LL-37 levels in GCF increase in the presence of gingival inflammation [12]. Along similar lines, recent development of highly sensitive biochemical tests has enabled tuned comparisons of inflammatory mediator levels in whole saliva and GCF samples between healthy control and individuals with periodontitis. However, most studies still focus on pro-inflammatory cytokines (e.g., IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-12, IL-17, TNF) [13–16], generally outnumbered in healthy subjects. On the contrary, there are fewer data on anti-inflammatory cytokines (e.g., IL-4, IL-9, IL-10, IL-13)[17], and besides, conclusions stay contradictory for some of them [1, 15].

Gaining a clear understanding of the mechanisms that underlie a pathological condition can help decide the optimal therapeutic strategy. In this respect, mechanical debridement by scaling and root planing (SRP) is considered the gold standard treatment for most patients with periodontitis [17]. Although overall it is an effective method, pain and dental fear have been reported to be associated with the procedure [18, 19]. In addition, it may be necessary to implement other adjuvant therapies (e.g., systemic and local antibiotics, laser-supported therapy, antimicrobial photodynamics) to increase the effectiveness of the treatment in patients refractory to SRP [20, 21]. The reason for this variable efficacy is uncertain but treated periodontal pockets have been suggested to be rapidly re-colonized by periodontal pathogens from yet-untreated periodontal pockets and oral niches [22, 23]. This implies that in some patients, conventional non-surgical periodontal therapy will not be efficient for long and the periodontal disease will turn into a recurrent condition.

Given the multifactorial nature of periodontal disease, great efforts are being addressed to identifying links between local, systemic, and environmental factors in the efficacy of non-surgical periodontal therapy [17]. Added to the stage severity of the disease and to the cytokine profile *in situ*, poor oral hygiene, the type of medication, smoking, obesity and diabetes among others have all been described to boost the development of the disease and to contribute to therapy failure [17, 24, 25]. Inversely, systemic diseases can also arise from poor periodontal health (e.g., diabetes, cardiovascular disease, Alzheimer's disease, pathological pain) [24, 26]. Demographic variables such as sex-dimorphism and age may also be taken into consideration in the treatment and management of periodontitis for it has been documented that men account for a higher prevalence compared to women [27] and older compared to young people [28].

As a corollary, the aim of the present cohort study was to investigate possible SRP treatment-derived shifts in personalized antimicrobial peptide (LL-37) and cytokine (IL-4, IL-6 and IL-10) profiles in GCF along the stages of periodontal disease and the effect that demographic and oral health habits may have on

these mediators' levels and on the outcome of the treatment. Understanding the mechanisms responsible for the variation of the therapeutic efficacy will help to achieve a better treatment and hence prognosis of the disease, and to determine if adjuvant experimental procedures may be needed.

2. Materials And Methods

2.1 Experimental design.

The present study aimed to evaluate if the antimicrobial peptide LL-37, cytokines IL-4, IL-10 and IL-6 together with volume of crevicular fluid and total protein concentration in crevicular fluid could be used as correlative biomarkers for the stage severity in periodontitis as well as prognostic factors in the management of the disease.

The study was designed as a prospective cohort, five-arm parallel-group study with a roughly one-and-a-half-month follow-up period. All participants were carefully examined by a same examiner (DM) and distributed according to their periodontal status into the different experimental groups. Along with periodontal examination, gingival crevicular fluid (GCF) sampling, self-reported pain measurements and a detailed medical history were taken, as well as information on their age, sex, the type of toothbrush used (manual/electric), number of teeth brushing per day and smoking habit. Periodontitis groups received full-mouth supra- and sub-gingival scaling and root planing (SRP). Comprehensive oral hygiene instructions were additionally given, which included the use of 0.12% chlorhexidine oral rinse twice daily for the following seven post-SRP days. Re-examination and new sample collection were carried out after 4–6 weeks. Total protein quantification in GCF and ELISA assays were performed by another examiner (MMG). Triple blinding was ensured to avoid any bias in the study. To do so, all images and samples were labeled with an alphanumeric identifier. Numbers corresponded to individual participants allocated in order of registration, whereas two different letters were assigned based on pre-SRP or post-SRP therapy. Medical and dental histories of each patient were only assigned a number to be referred by.

2.2. Participants.

A total of 60 individuals (33 males and 27 females; with age range 23–77 years old; mean age 42.20 (SD 15.76)) were recruited from the University Clinic at the Faculty of Health Sciences of Universidad Rey Juan Carlos between June 2021 and June 2022 following a screening evaluation including full-mouth periodontal probing and radiographic examination. The inclusion criteria were aged 18 or over, with no previous history of periodontal treatment in the last six months and no medical or dental history that could contraindicate periodontal treatment. Exclusion criteria included current pregnancy or lactation, immunological disorders, periodontal treatment within the last 6 months, oral contraception and antibiotic usage within the last 3 months, and infections (such as HIV, hepatitis, and tuberculosis). In addition, third molar teeth were not considered for GCF sampling for being often impacted or previously extracted. After fully explaining the study's aim, all subjects gave informed written consent.

The healthy control group originally consisted of 30 people (10 women and 20 men; age range 25–74; mean age 31.30 (SD 9.90)). Likewise, the patient group was formed of 30 individuals (17 women and 13 men; age range 23–77; mean age 53.10 (SD 12.71)) covering periodontitis stage I, II, III and IV. For the molecular assays, to maintain consistent numbering in all groups samples from only 15 subjects of the healthy control group were analyzed (7 women and 8 men; age range 25–74; mean age 31.87 (SD 12.72)), whereas the patient group was further subdivided in two groups of 15 subjects each: periodontitis stage I-II (8 women and 7 men; age range 23–77; mean age 54.27 (SD 14.42)) and III-IV (9 women and 6 men; age range 34–69; mean age 51.93 (SD 11.13)). A flow-diagram of the selection criteria is shown in Fig. 1. All study procedures were performed in compliance with a protocol approved by the Ethical Committee of Universidad Rey Juan Carlos (protocol number 2711201916719) and followed STROBE guidelines for reporting observational studies.

2.3. Determination of periodontal status.

Periodontal examination and assessment were performed according to the parameters proposed in the Classification of Periodontal and Peri-implant Diseases and Conditions presented at the 2017 World Workshop by the American Academy of Periodontology (AAP) and the European Federation of Periodontology (EFP) in Chicago, IL, USA [29]. For each patient a unique periodontitis entity supported on a full-mouth periodontal chart was recognized, with four stage parameters that included probing depth (PD), interdental clinical attachment level (CAL), plaque index (PI), and bleeding on probing (BOP) measurements. Three grades were additionally used as indicators of the progression rate of the periodontal disease. A full periodontal examination (6 point pocket chart) was performed with a Williams periodontal probe (Hu-Friedy Mfg. Co., LLC; Chicago, IL, USA; PW6). Based on these measurements, a periapical radiographic series was performed for those indivuals suspected of periodontitis to determine periodontal bone loss (BL).

Briefly, the root was divided into three parts from the cement-enamel junction (CEJ) to the root apex: coronal third, middle third and apical third. *Stage I* was conceived as the initial form of the disease, with interdental CAL = 1-2mm, PD ≤ 4mm, radiographic interdental BL affecting the coronal third (< 15%) and no tooth loss; *Stage II* was considered a mild form of the disease, with CAL = 3-4mm, PD ≤ 5mm, BL affecting the coronal third (15–33%) and no tooth loss; *Stage III* reflected a moderate form of the periodontal disease with potental risk of tooth loss, CAL ≥ 5mm, PD ≥ 6mm, BL extending from the coronal third to the median third (33–50%) and < 4 teeth loss; *Stage IV* represented the most severe form of periodontitis with potential risk of losing all remaining teeth, CAL ≥ 5mm, PD ≥ 6 mm, BL from the middle third to the apical third (> 50%), > 5 teeth loss and with or without loss of masticatory function.

Grades were used as indicators of the progression rate of periodontitis based on the severity, complexity and type of periodontitis progression as follows. *Grade A*: slow progression, absence of attachment or bone loss in the last five years, heightened plaque or biofilm levels and scarce bone loss. *Grade B*: moderate progression, BL < 2 mm in the last five years and bone destruction in agreement with the plaque levels. Additionally, if the patient was a smoker on average daily cigarette consumption under 10 or were they diabetic with glycated haemoglobin levels (HbA1c) < 7%, they also entered this category. *Grade C*: rapid progression, BL > 2mm in the last five years, low plaque levels in relation to the amount of bone loss and patterns that suggest rapid progression or an

early onset of the disease. Additionally, all patients with a high cigarette consumption (over 10 cigarettes per day) or diagnosed with diabetes with HbA1c > 7% were included in this category.

Alternatively, the bone loss/age (BL/A) ratio calculated from full-mouth pericapical radiographs was used for grading patients with no previous periodontal records in the past 5 years (*Grade A* < 0.25, *Grade B* = 0.25 to 1.0, *Grade C* > 1.0). In addition to this, heavy smokers (smoking more than 10 cigarretes per day) were systematically graded as *C*, whilst moderate smokers (those who smoke less than 10 cigarretes per day) were classified as *Grade B*. Furthermore, diabetic patients with HbA1c < 7.0 were rated as *Grade B*, whereas those with HbA1c ≥ 7.0 were upgraded to *C*. Distinctions made for smokers and HbA1c rely on the fact that smoking and diabetes have been established as two major risk factors for periodontitis [28, 30]. Overall, grade increased proportionally to the stage of periodontitis (Supplementary Table 1).

2.4. Evaluation of Pain.

Pain was evaluated using an 11-point self-reported numeric pain rating scale (NPRS) [31]. A 0–10 score, in which 0 represented “no pain” and 10 indicated “the most pain imaginable”, was recorded from the participants, who verbally selected the value that was most in line with the intensity of oral pain that they had experienced during periodontal probing. NPRS was implemented on first attendance (pre-SRP) and on the re-examination day.

2.5. Gingival crevicular fluid sampling and processing.

Gingival crevicular fluid was sampled and processed as previously described [32] with minor modifications. Briefly, supragingival plaque was carefully removed before GCF sampling. Collection sites were then isolated using cotton rolls and dried with air jets, and corresponded to those showing greatest clinical signs of inflammation and highest CAL along with intraoral periapical radiographic confirmation of bone loss. In this regard, all teeth surfaces (buccal, mesio-buccal and disto-buccal) were more or less similarly represented within and between the different study groups (Supplementary Table 2). PerioPaper® GCF collection strips (Oraflow Inc; Hewlett, NY, USA; 593520) were overlaid and placed at the gingival crevice region until mild resistance was felt. Then, left in place for 30 s to prevent any mechanical irritation. Any strip contaminated with blood was excluded.

The volume was calculated with the help of a Periotron® 8010 (Oraflow Inc; Hewlett, NY, USA; 593480) device according to the supplier’s indications and corresponded to an inflammatory exudate that was used as an index for the degree of periodontal inflammation. The readings (μl/h) were converted to an actual volume (μl) by reference to the standard curve[13]. The same representative periodontal pocket from patients with periodontitis stage I-II or III-IV was compared pre-SRP and post-SRP and to samples obtained from healthy individuals.

Immediately after sample collection, the strips were stored in sterile test tubes (Eppendorf Corporate; Hamburg, Germany; 0030125150) with an added volume of 500 μl of 1x phosphate buffered-saline (PBS, pH 7.2) and refrigerated at 4 ºC until the following day. A vortex-type stirrer was used to thoroughly mix the exudate with the dilutant for 2min and the sample was then centrifuged at 10,000 rpm for 10 min. Subsequently, the paper strips were removed and the remaining volumes preserved at -80 ºC until their use.

Additionally, total protein concentration for each sample was measured in duplicate or triplicate as required using the NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) following the supplier’s instructions. Fresh prepared 1x PBS (pH 7.2) was used as sample blank.

2.6. Quantification of cathelicidin LL-37 and cytokines IL-4, IL-6, IL-10.

Commercially available human antibacterial peptide LL-37 enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Technology LLC; Houston, TX, USA; CSB-E14948h) and IL-4, IL-6 and IL-10 high sensitivity ELISA kits (Diaclone SAS; Besançon, France; 850.890, 950.035 and 850.880, respectively) were used to determine the concentration of each compound. Assays were carried out in accordance with the manufacturers’ instructions and all measurements were done in duplicate. The results were expressed as total amount (pg) of cathelecidin LL-37 or cytokine per site and also as normalized to total protein content in GCF (pg/μg). Samples with protein levels below the assay’s detection limit were scored as 0.

2.7. Statistical analyses.

Sample size was calculated using a conservative method of power analysis (G*Power 3.1.9.4, Heinrich-Heine-Universität Düsseldorf, Germany). Power calculations indicated a sample size of 27 participants to be necessary based on a two-tailed test with an effect of 0.50, a difference of 50% in average value and maximum standard deviation of 80% (alpha, 0.05, power, 0.90). Thus, 30 individuals per group (healthy control and patients) were enrolled in accounting for any potential complications.

All analyses were performed using statistical software (GraphPad Prism 8.0.1, San Diego, CA, USA). Age and tooth brushing frequency parameters were presented as means and standard desviations (SD). All the rest were shown as means and standard error of the mean (SEM). The normality of all data was assessed by the Shapiro-Wilk test. Two-way ANOVA (treatment x group) followed by Tukey’s or Sidak’s multiple comparisons test, as required, were used to determine statistical significance between baseline and post-SRP effects and between healthy control and periodontitis stage I-II and III-IV. The same analysis was also used to determine statistical significance between men and women (sex x group). One-way ANOVA followed by Tukey’s multiple comparisons test was used to analyze the effect of age or oral hygiene (number of teeth brushing per day) on healthy/periodontitis stage in the total sample, whereas two-way ANOVA (sex x group) followed by Sidak’s multiple comparisons test was used to analyze the effect of age or oral hygiene on healthy/periodontitis stage in men and women separately. Additionally, simple linear regression analyses were conducted to predict whether there was a linear relationship between age, oral hygiene (number of teeth brushing per day and type of toothbrush), diabetes or smoking and healthy/periodontitis stage. To study the possible correlations between each molecular mediator and pain, two-tailed Spearman’s correlation analyses were used. In all statistical analysis, a level of significance of $P < 0.05$ was assumed.

3. Results

3.1. Periodontal status and clinical effects of periodontal therapy.

The mean PD value of periodontitis stage I-II patients at baseline (4.53 ± 0.13) was significantly lower ($p < 0.0001$) than the mean PD of periodontitis stage III-IV group (6.47 ± 0.39). This was also true for CAL (5.20 ± 0.22 vs. 8.40 ± 0.51, $p < 0.0001$) and percentage of sites with PD > 4 mm (12.40 ± 3.35 vs. 47.93 ± 6.13, $p < 0.0001$). Four-six weeks after SRP therapy, all three said parameters were lowered in both periodontitis Stage I-II (PD: 3.40 ± 0.21, $p < 0.05$; CAL: 3.80 ± 0.28, $p < 0.05$; PD > 4 mm: 7.00 ± 2.61) and III-IV groups (PD: 5.27 ± 0.36, $p < 0.05$; CAL: 7.67 ± 0.46; PD > 4 mm: 39.13 ± 7.12). Mean values for all three parameters were significantly lower ($p < 0.0001$) in periodontitis Stage I-II patients as compared to Stage III-IV also post-SRP (Table 1). No statistically significant differences were found between men and women, except for the percentage of sites with PD > 4mm in periodontitis stage III-IV patients post-SRP (55.50 ± 13.53 vs. 28.22 ± 5.86 for men and women, respectively, $p < 0.05$).

Table 1

Periodontal clinical parameters (mean ± SEM) of the sites sampled for molecular analyses in the two clinical groups, at baseline and 4–6 weeks after therapy. PD = probing depth; CAL = clinical attachment level; BOP = bleeding on probing; PI = plaque index. * $p < 0.05$ pre-SRP vs. post-SRP;

$p < 0.0001$ vs. Periodontitis Stage I-II. Two-way ANOVA followed by Sidak's multiple comparisons test.

	Stage I-II		Stage III-IV	
	Pre-SRP	Post-SRP	Pre-SRP	Post-SRP
Clinical parameters				
PD (mm)	4.53 ± 0.13	3.40 ± 0.21*	6.47 ± 0.39	5.27 ± 0.36*,
CAL (mm)	5.20 ± 0.22	3.80 ± 0.28*	8.40 ± 0.51	7.67 ± 0.46
% of sites with				
PD > 4 mm	12.40 ± 3.35	7.00 ± 2.61	47.93 ± 6.13	39.13 ± 7.12
BOP	43.00 ± 7.69	19.67 ± 4.65*	62.07 ± 7.52	37.67 ± 6.20*
PI	33.67 ± 6.13	16.73 ± 3.10	47.93 ± 7.69	27.80 ± 4.84*

Percentage of sites with BOP and PI also significantly decreased after periodontal treatment in both periodontitis Stage I-II (BOP: 43.00 ± 7.69 vs. 19.67 ± 4.65, $p < 0.05$; PI: 33.67 ± 6.13 vs. 16.73 ± 3.10) and III-IV patients (BOP: 62.07 ± 7.52 vs. 37.67 ± 6.20, $p < 0.05$; PI: 47.93 ± 6.13 vs. 27.80 ± 4.84, $p < 0.05$). However, differences between periodontitis Stage I-II and III-IV were not found to be statistically significant. No statistically significant differences were either found between men and women (data not shown).

3.2. Evaluation of pain.

The mean NPRS scores were 1.47 ± 0.12, 3.60 ± 0.40 and 6.00 ± 0.53 for healthy control, periodontitis Stage I-II and III-IV groups, respectively. Statistically significant differences existed between healthy and patient groups ($p < 0.0001$) and between patient groups ($p < 0.0001$). These values decreased significantly after SRP treatment in both patient groups ($p < 0.0001$). Furthermore, there were no statistically significant differences post-SRP as compared to healthy control for either stage (I-II: 1.73 ± 0.25 and III-IV: 2.27 ± 0.23) (Fig. 2a).

When sex differences were examined similar NPRS scores were observed for women (1.40 ± 0.27) and men (1.50 ± 0.14) in the healthy group, but statistically significant differences were encountered in periodontitis Stage I-II ($p < 0.01$) and III-IV ($p < 0.05$) groups. Intriguingly, women reported higher scores than men in mild (4.50 ± 0.57 vs. 2.57 ± 0.20) but not in moderate-severe (5.33 ± 0.62 vs. 7.00 ± 0.86) stages. NPRS scores were reduced in both sexes post-SRP (Stage I-II: $p < 0.001$, ns for men though; Stage III-IV: $p < 0.0001$) but with no significant differences between them (Stage I-II: 2.13 ± 0.40 vs. 1.29 ± 0.18; Stage III-IV: 2.00 ± 0.24 vs. 2.67 ± 0.42) (Fig. 2b).

3.3. GCF volume and total protein concentration.

GCF volume was statistically significant lower in healthy control (0.11 ± 0.04) than in periodontitis Stage I-II (0.65 ± 0.15, $p < 0.0001$) and III-IV (0.88 ± 0.10, $p < 0.0001$) groups, with no significant differences between patient groups. These volumes decreased following SRP treatment (I-II: 0.52 ± 0.15, ns; III-IV: 0.39 ± 0.11, $p < 0.01$), particularly in Stage III-IV periodontitis group, which showed no statistically significant differences vs. healthy control unlike Stage I-II ($p < 0.01$) (Fig. 3a). No statistically significant differences were found between sexes and periodontitis groups compared to healthy control (women: 0.10 ± 0.06: men: 0.12 ± 0.05) followed the same trend as when computing both sexes together: Stage I-II (pre-SRP: 0.58 ± 0.21, ns for women and 0.72 ± 0.23, $p < 0.05$ for men;

post-SRP: 0.74 ± 0.24, $p < 0.01$ for women and 0.28 ± 0.13, ns for men) and III-IV (pre-SRP: 0.90 ± 0.15, $p < 0.001$ for women and 0.85 ± 0.11, $p < 0.01$ for men; post-SRP: 0.44 ± 0.16 and 0.31 ± 0.13 for women and men respectively, ns) (Fig. 3b).

At baseline, total protein concentration in GCF (µg/ml) was higher for periodontitis groups, 44.79 ± 7.88 (Stage I-II) and 69.93 ± 9.50 (Stage III-IV), than for the healthy control group, 30.41 ± 3.55. Moreover, statistically significant differences were found between Stage III-IV and healthy control groups ($p < 0.01$). Although not statistically significant, 4–6 weeks after SRP treatment, total protein concentration in GCF was reduced as compared to baseline in both groups (Stage I-II: 35.70 ± 6.50, Stage III-IV: 47.16 ± 10.28) (Fig. 3c). When sex influence was analyzed, no significant differences were found between women and men at baseline: healthy control (women 30.31 ± 4.08, men: 30.50 ± 5.89), Stage I-II (women: 44.06 ± 11.00, men: 45.75 ± 12.26), Stage III-IV (women: 60.17 ± 13.94, men: 79.10 ± 7.98). Protein concentration was also similar for both sexes after SRP treatment: Stage I-II (women: 36.13 ± 9.34, men: 35.21 ± 9.72), Stage III-IV (women: 36.63 ± 8.69, men: 61.20 ± 20.76). It was also noticeable that protein levels increased according to the severity of the periodontal disease in both women and men; however, differences against healthy control were only evident for men with periodontitis Stage III-IV ($p < 0.05$). Following treatment, protein concentration decreased in both sexes but again, these observations were not statistically significant (Fig. 3d).

3.4. Levels of antimicrobial peptide LL-37, pro/anti-inflammatory cytokine IL-6 and anti-inflammatory cytokines IL-4 and IL-10 in GCF.

The mean absolute LL-37 levels in GCF were 9.96 ± 6.13 pg, 32.62 ± 10.49 pg and 26.47 ± 12.95 pg for healthy control, periodontitis Stage I-II and III-IV groups, respectively. Statistically significant differences were found between healthy and periodontitis Stage I-II group ($p < 0.05$). After SRP treatment values for periodontitis State I-II and III-IV were 3.20 ± 2.60 pg and 17.16 ± 9.15 pg, respectively. Such reduction in LL-37 levels was only statistically significant for periodontitis Stage I-II ($p < 0.05$) though (Fig. 4a). In a similar way, normalized levels of LL-37 to total protein in GCF were 0.08 ± 0.06 pg/µg, 1.30 ± 0.46 pg/µg and 0.51 ± 0.26 pg/µg for healthy control, periodontitis Stage I-II and III-IV groups. Significant statistically differences as compared to healthy control individuals were only evident for periodontitis Stage I-II patients ($p < 0.001$). Such levels were significantly increased as compared to Stage III-IV ($p < 0.05$). Following SRP treatment, normalized LL-37 levels decreased for both periodontitis Stage I-II (0.09 ± 0.06 pg/µg, $p < 0.001$) and Stage III-IV (0.14 ± 0.08 pg/µg, ns), close to healthy control. (Fig. 4c).

When sex influence was examined no statistically significant differences were observed between women and men for any of the healthy control (W: 0.00 ± 0.00 pg; M: 16.94 ± 10.02 pg), periodontitis Stage I-II (W: 18.20 ± 9.12 pg; M: 49.11 ± 18.81 pg) or Stage III-IV (W: 34.46 ± 20.33 pg; M: 13.17 ± 6.68 pg) groups, although it was noticeable that LL-37 levels for men under Stage I-II were higher than under Stage III-IV. No statistically significant differences were observed following SRP treatment either for periodontitis Stage I-II (W: 5.18 ± 5.18 pg; M: 1.22 ± 1.03 pg) and Stage III-IV (W: 25.75 ± 13.06 pg; M: 0.00 ± 0.00 pg) (Fig. 4b). Normalized LL-37 levels to total protein in GCF showed a similar trend, with no significant differences between women and men for healthy control (W: 0.00 ± 0.00 pg/µg; M: 0.14 ± 0.10 pg/µg), periodontitis Stage I-II (W: 0.84 ± 0.60 pg/µg; M: 1.82 ± 0.70 pg/µg) or Stage III-IV (W: 0.75 ± 0.40 pg/µg; M: 0.11 ± 0.06 pg/µg) groups. However, men under periodontitis Stage I-II showed statistically significant increased levels as compared to healthy control ($p < 0.01$) and to Stage III-IV ($p < 0.05$). Following SRP treatment levels in periodontitis Stage I-II (W: 0.12 ± 0.12 pg/µg; M: 0.05 ± 0.04 pg/µg) and Stage III-IV (W: 0.13 ± 0.10 pg/µg; M: 0.15 ± 0.15 pg/µg) were decreased, although only reductions for men under Stage I-II proved to be statistically significant ($p < 0.01$) (Fig. 4d).

Dual anti/pro-inflammatory cytokine IL-6 followed similar trends to antimicrobial peptide LL-37. IL-6 levels in GCF (pg/site) were increased for both periodontitis Stage I-II (590.30 ± 27.10 pg) and III-IV (759.20 ± 82.11 pg) as compared to control group (513.90 ± 52.74 pg) at baseline, although no significant statistical differences were found. However, statistically significant differences were observed between both patient groups ($p < 0.05$). Similarly, IL-6 showed opposite trends to those of IL-4 and IL-10 following SRP treatment. Absolute levels of IL-6 in GCF decreased after treatment (Stage I-II: 333.50 ± 21.27 pg; Stage III-IV: 395.10 ± 51.27 pg), although no evident significant statistical differences were encountered vs. healthy control or compared to baseline levels. Neither between patient groups (Fig. 5a). IL-6 normalized values to total protein in GCF were 15.05 ± 2.05 pg/µg, 20.26 ± 3.12 pg/µg and 10.42 ± 1.74 pg/µg for healthy control, periodontitis Stage I-II and III-IV groups, respectively. Significant statistical differences were only found between healthy control and periodontitis Stage III-IV groups ($p < 0.01$). IL-6 levels following SRP treatment were ambiguous: they decreased in periodontitis Stage I-II (14.18 ± 2.75 pg/µg, $p < 0.01$) but increased in Stage III-IV (13.19 ± 3.42 pg/µg, $p < 0.0001$) patients (Fig. 5c).

IL-4 and IL-10 alike, no significant differences were found between women and men for IL-6. Stage III-IV absolute levels (W: 835.90 ± 114.30 pg, $p < 0.05$; M: 605.70 ± 56.63 pg, ns) but not Stage I-II (W: 355.60 ± 31.98 pg, ns; M: 314.50 ± 28.64 pg, ns) were increased as compared to healthy control individuals (W: 508.30 ± 61.41 pg; M: 518.40 ± 84.29 pg). Levels decreased following treatment for both periodontitis Stage I-II (W: 355.60 ± 31.98 pg; M: 314.50 ± 28.64 pg) and Stage III-IV (W: 430.70 ± 80.86 pg, $p < 0.001$; M: 341.70 ± 41.94 pg), however significant statistical differences against baseline were only encountered for women under periodontitis Stage III-IV (Fig. 5b). When analyzing normalized values in women and men separately, no statistically significant differences were found for any of the comparisons carried out (Fig. 5d).

Unlike antimicrobial peptide LL-37 and IL-6, anti-inflammatory cytokines IL-4 and IL-10 levels in GCF were substantially decreased in the periodontitis groups as compared to healthy control. The mean absolute IL-4 levels in GCF were 346.30 ± 18.34 pg, 144.90 ± 22.68 pg and 15.80 ± 6.51 pg for healthy control, periodontitis Stage I-II and III-IV groups, respectively. Statistically significant differences existed between healthy and patient groups ($p < 0.0001$) and between patient groups ($p < 0.0001$). These values remained unvaried after SRP treatment in both patient groups (Stage I-II: 178.30 ± 17.83 pg; Stage III-IV: 29.35 ± 10.16 pg) (Fig. 6a). Normalized levels of IL-4 to total protein in GCF showed similar trends. There were statistically significant differences ($p < 0.0001$) between healthy (10.67 ± 1.13 pg/µg) and periodontitis Stage I-II (4.27 ± 0.92 pg/µg) and III-IV (0.66 ± 0.42 pg/µg) groups. Differences between both patient groups remained noticeable ($p < 0.05$). Following SRP treatment similar trends were observed for both groups, with statistically significant differences as compared to healthy control for either periodontitis stage (I-II: 6.63 ± 1.12 pg/µg, $p < 0.01$; and III-IV: 0.78 ± 0.29 pg/µg, $p < 0.0001$) and between patient groups ($p < 0.0001$) (Fig. 6c).

When sex influence was examined similar absolute IL-4 levels were observed for women and men in the healthy (W: 359.50 ± 28.40 pg; M: 335.80 ± 24.78 pg), periodontitis Stage I-II (W: 133.10 ± 30.67 pg; M: 158.40 ± 35.51 pg) and Stage III-IV (W: 16.45 ± 7.39 pg; M: 14.83 ± 12.84 pg) groups. Statistically significant differences existed between healthy and patient groups ($p < 0.0001$); however, statistically significant differences between patient groups were only evident for women ($p < 0.01$). Again, following SRP treatment no statistically significant differences were found between women and men in any of the periodontitis Stage I-II (W: 164.20 ± 25.53 pg; M: 190.30 ± 25.75 pg) nor Stage III-IV (W: 28.45 ± 11.43 pg; M: 30.84 ± 20.82 pg) groups, but were still encountered vs. the healthy control group (Stage I-II W/M: $p < 0.0001$ / $p < 0.001$; Stage III-IV W/M: $p < 0.0001$) and between groups (W: $p < 0.01$; M: $p < 0.001$) (Fig. 6b). Normalized IL-4 to total protein in GCF also showed similar levels between women and men in healthy (W: 12.12 ± 1.42 pg/µg; M: 9.21 ± 1.69 pg/µg), periodontitis Stage I-II (W: 4.53 ± 1.20 pg/µg; M: 3.97 ± 1.52 pg/µg) and Stage III-IV (W: 0.97 ± 0.69 pg/µg; M: 0.19 ± 0.17 pg/µg) groups. Statistically significant differences existed between healthy and patient groups for women (Stage I-II: $p < 0.001$; Stage III-IV: $p < 0.0001$) and men (Stage I-II: $p < 0.05$; Stage III-IV: $p < 0.0001$), but not between patient groups. One and a half months after SRP similar reduced trends vs. healthy control individuals were found (Stage I-II: 6.20 ± 1.78 pg/µg, $p < 0.01$, and 7.05 ± 1.47 pg/µg, ns for women and men, respectively; Stage III-IV: 0.72 ± 0.30 pg/µg, $p < 0.0001$, and 0.89 ± 0.65 pg/µg, $p < 0.001$). Statistically significant differences were found between both patient groups for women and men ($p < 0.05$) (Fig. 6d).

The mean absolute IL-10 levels in GCF were 127.20 ± 8.43 pg, 50.76 ± 7.42 pg and 13.49 ± 3.63 pg for healthy control, periodontitis Stage I-II and III-IV groups, respectively. Evident statistically significant differences existed between healthy and patient groups ($p < 0.0001$) and between patient groups ($p < 0.01$). These values remained unvaried after SRP treatment in periodontitis Stage I-II group (77.11 ± 7.82 pg); however, levels for Stage III-IV significantly increased post-SRP (40.06 ± 6.70 pg, $p < 0.05$) (Fig. 7a). Normalized levels of IL-10 to total protein in GCF showed similar trends. There were statistically significant differences ($p < 0.0001$) between healthy (4.96 ± 0.79 pg/µg) and periodontitis Stage I-II (1.35 ± 0.26 pg/µg) and III-IV (0.19 ± 0.05 pg/µg) groups, although differences between both patient groups were not statistically noticeable. Following SRP treatment similar trends were observed for both groups, with statistically significant differences as compared to healthy control for either periodontitis stage (I-II: 2.48 ± 0.29 pg/µg, $p < 0.01$; and III-IV: 1.10 ± 0.20 pg/µg, $p < 0.0001$) (Fig. 7c).

When sex influence was examined similar absolute IL-10 levels were observed for women and men, with statistically significant differences between periodontitis Stage I-II (W: 45.66 ± 8.90 pg, $p < 0.0001$; M: 56.57 ± 12.62 pg, $p < 0.001$) and Stage III-IV (W: 15.43 ± 5.07 pg, $p < 0.0001$; M: 10.59 ± 5.24 pg, $p < 0.0001$) groups as compared to healthy control individuals (W: 131.70 ± 11.28 pg; M: 123.50 ± 12.61 pg). Statistically significant differences between patient groups were only evident for men ($p < 0.05$). Again, following SRP treatment no statistically significant differences were found between women and men in any of the periodontitis Stage I-II (W: 76.27 ± 10.38 pg; M: 78.08 ± 12.71 pg) nor Stage III-IV (W: 45.45 ± 9.20 pg; M: 31.97 ± 9.45 pg) groups, but were still encountered vs. the healthy control group (Stage I-II W/M: $p < 0.01$ / $p < 0.05$; Stage III-IV W/M: $p < 0.0001$) and between groups but only for men ($p < 0.05$) (Fig. 7b). Normalized IL-10 to total protein in GCF also showed similar levels between women and men in healthy (W: 4.63 ± 0.77 pg/µg; M: 5.23 ± 1.32 pg/µg), periodontitis Stage I-II (W: 1.40 ± 0.34 pg/µg; M: 1.29 ± 0.43 pg/µg) and Stage III-IV (W: 0.23 ± 0.07 pg/µg; M: 0.12 ± 0.06 pg/µg) groups. Statistically significant differences existed between healthy and patient groups for women (Stage I-II: $p < 0.01$; Stage III-IV: $p < 0.0001$) and men (Stage I-II: $p < 0.001$; Stage III-IV: $p < 0.0001$), but not between patient groups. One and a half months after SRP similar reduced trends vs. healthy control individuals were found (Stage I-II: 2.31 ± 0.44 pg/µg, ns, and 2.66 ± 0.40 pg/µg, $p < 0.05$ for women and men, respectively; Stage III-IV: 1.22 ± 0.29 pg/µg, $p < 0.01$, and 0.88 ± 0.27 pg/µg, $p < 0.001$) (Fig. 7d).

Secondly, the association between pain and the molecular mediators was further addressed. Distribution of all molecular mediators (pg or pg/µg) departed significantly from normality. Therefore, two-tailed Spearman's correlation analyses were used to analyze the associations between NPRS scores and the aforementioned parameters. Correlations were found for all two set of variables, except for NPRS and IL-6 (pg/µg) (Table 2). As pain scores increased, so did LL-37 (pg), LL-37 (pg/µg) and IL-6 (pg) levels in GCF (r = 0.3125, 0.3185 and 0.4284, respectively). On the contrary, IL-4 (pg), IL-4 (pg/µg), IL-10 (pg) and IL-10 (pg/µg) levels decreased accordingly (r = -0.4743, -0.4579, -0.5194 and − 0.6042, respectively).

Table 2
Spearman's rank correlation between NPRS score and the molecular mediators LL-37, IL-6, IL-4 and IL-10. r, correlation coefficient; 95% CI, 95% confidence interval.

Non-parametric Spearman's correlation			
	95% CI	r(df)	*p*
LL-37 (pg)	0.0767–0.5153	r(68) = 0.3125	< 0.01 (**)
LL-37 (pg/µg)	0.0851–0.5188	r(69) = 0.3185	< 0.01 (**)
IL-6 (pg)	0.2047–0.6096	r(66) = 0.4284	< 0.001 (***)
IL-6 (pg/µg)	-0.4136–0.0684	r(64) = -0.1836	0.140 (ns)
IL-4 (pg)	-0.6402 – -0.2661	r(70) = -0.4743	< 0.001 (***)
IL-4 (pg/µg)	-0.6288 – -0.2449	r(69) = -0.4579	< 0.001 (***)
IL-10 (pg)	-0.6733 – -0.3224	r(71) = -0.5194	< 0.001 (***)
IL-10 (pg/µg)	-0.7357 – -0.4290	r(71) = -0.6042	< 0.001 (***)

3.5. Association between risk factors and the clinical condition.

Information on the age, oral hygiene practices (tooth brushing frequency and type of toothbrush), diabetes and smoking habits is displayed in Table 3. As compared to healthy control (31.87 (SD 12.72), the age of patients from the periodontitis Stage I-II (59.14 (SD 7.99)) and III-IV (52.71 (SD 12.88)) groups was significantly higher ($p < 0.0001$ and $p < 0.001$, respectively). This was also true when women and men were analyzed separately: healthy control (W: 29.29 (SD 7,41); M: 34.12 (SD 16.25)), periodontitis Stage I-II (W: 59.75 (SD 8.30), $p < 0.001$; M: 58.33 (SD 9.29), $p < 0.05$) and III-IV (W: 53.20 (SD 9.73), $p < 0.01$; M: 51.50 (SD 24.75)).

Table 3
Characteristics of the sample according to common risk factors associated to periodontitis. Data are expressed as mean (SD). $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$, $^{++++}$ $p < 0.0001$ vs. healthy control. One-way or two-way ANOVA (sex x group) followed by Tukey's multiple comparisons test. M = manual; E = electric.

	Healthy			Stage I-II			Stage III-IV		
	women	men	sum	women	men	sum	women	men	sum
age (y) (mean (SD))	29.29 (7.41)	34.13 (16.25)	31.87 (12.72)	59.75 (8.30)$^{+++}$	58.33 (9.29)$^{+}$	59.14 (7.99)$^{++++}$	53.20 (9.73)$^{++}$	51.50 (24.75)	52.71 (12.88)$^{+++}$
Teeth brushing/day (mean (SD))	2.57 (0.53)	2.75 (0.46)	2.67 (0.49)	2.25 (0.71)	2.57 (0.53)	2.40 (0.63)	2.22 (0.67)	2.00 (0.63)	2.13 (0.64)$^{+}$
Type of toothbrush (n) (M/E)	0/7	2/6	2/13	5/3	5/2	10/5	5/4	4/2	9/6
Diabetes (n)	0	0	0	0	0	0	0	1	1
Smoker (n)	0	1	1	2	0	2	0	3	3

On initial univariate analysis, older people were more likely to suffer from a more severe stage of periodontitis ($F(1,43) = 15.38$, $p < 0.001$), with $R^2 = 0.26$. So did individuals who brushed their teeth less frequently ($F(1,43) = 6,26$, $p < 0.05$) with $R^2 = 0.13$ and those making use of a manual toothbrush ($F(1,43) = 7.34$, $p < 0.01$), with $R^2 = 0.15$. No association was found however between the severity of periodontitis and diabetes ($F(1,43) = 1.52$, $p = 0.225$), with $R^2 = 0.03$, or smoking habits ($F(1,43) = 1.13$, $p = 0.293$), with $R^2 = 0.03$.

4. Discussion

Periodontal disease represents a public health concern due to its high prevalence and uncertain recurrence after conventional non-surgical treatment. In addition, therapy outcomes may be variable; however, the subjacent cause is yet to be unveiled [33, 35]

The results of the current study indicate that clinical periodontal indexes PD, CAL, BOP and PI were higher for periodontitis stage III-IV than for stage I-II and improved in both periodontitis groups 4–6 weeks after receiving conventional non-surgical periodontal treatment. These findings concur with other studies stating that SRP is to be considered a keystone in the initial management of periodontal disease [20, 21]. However, it is worth mentioning that follow-up studies are scarce in this field, which may account for the unsolved problem of the condition regarding its chronicity and recurrence despite the initial treatment efficacy. Additionally, although no statistically significant differences were found between sexes for the aforementioned parameters, men did present a greater number of pockets deeper than 4 mm than women ($p < 0.05$). In this regard, we consider that including this form of presentation for the probing depth parameter must be taken into consideration in future works, for most studies avoid its use despite the complementary information it is herein proved to provide.

As previously stated, periodontitis may cause orofacial pain or periodontal discomfort[26] and pain has been reported to be associated with SRP [18, 19]. In this study, a NPRS was used to determine the relationship among pain to dental probing and periodontal status. Although unidimensional pain scale scores are supposed to reflect the intensity of the somatosensory aspects of physical pain, they are also related to the intensity of emotional aspects and therefore constitute one of the most reliable and valid measurement tools for self-report of pain. Moreover, they are easy to use and simple to describe [36]. At baseline, NPRS scores were higher for the periodontitis than for the control group, especially in stage III-IV. Interestingly women expressed higher pain scores than men in mild but not in severe stages of periodontitis. A possible explanation could be that steroid hormones may be responsible for differences in pain sensation between women and men and could be involved in the periodontitis process itself [37]. In fact, sexual dimorphism has previously been suggested to be implicated modifying the bacterial biofilm and hence the host immune response at the periodontal tissue [27], which in turn could signify higher pain sensitivity. However, we are uncertain to the exact reasons women reported higher scores in mild periodontitis stages whereas men in severe. After 4–6 weeks following conventional non-surgical treatment, no evident differences were found between the NPRS scores of all groups. That is, SRP therapy reduced pain levels to those of a healthy control individual in both women and men. We next endeavored to evaluate the association of pain and a series of molecular mediators in the GCF.

As extensively reported [8, 12, 38–40], the quantification of GCF volume and the characterization of the mediators exuded in it are common methods aimed at assessing the severity of the disease. We first confirmed that, contrary to healthy controls, higher GCF volumes corresponded to patients with more severe

periodontitis [12] and decreased following SRP treatment to that of healthy control. These changes were independent of sex and the same profile was observed for total protein quantification in GCF, which implies that crevicular protein levels can also be used as reliable indicators of periodontitis severity in absence of the Periotron device.

Of all proteins that are present in the GCF, we focused on a series of “anti”-mediators. Like that reported by Türkoğlu et al. [12], we found increased levels of LL-37 (pg/site) in the presence of gingival inflammation. Although no evident differences were found between periodontitis Stage I-II and III-IV, relative values normalized to total protein concentration were greater for periodontitis Stage I-II than for III-IV ($p < 0.05$). However, this may lay on the basis that the amount of protein exuded in the GCF was greater in more severe stages of the disease and LL-37 may not represent much of it. In either case, 4–6 weeks post-SRP LL-37 levels were comparable to healthy control. Similar as explained for LL-37 was observed for the pro-/anti-inflammatory cytokine IL-6, in line to that previously reported by Yue et al. [15]. On the other hand, anti-inflammatory cytokines IL-4 and IL-10 showed a negative correlation with periodontitis, so that as the severity stage of the disease increased, levels of crevicular IL-4 and IL-10 decreased. This was in contradiction to that reported by Yue et al. [15] and Archana *et al.* [41], but in line with that shown by Pradeep et al. [42] and Varma et al. [1] for IL-4 and IL-10, respectively. Similarly, although Al-Hamoudi reported in 2020 [17] increased levels of IL-4 and IL-10 compared with their respective baseline values three months following SRP therapy, our study shows that SRP therapy had little or no effect on the levels measured at baseline. This controversy may however be explained by the different follow-up time that we used, since the trends in both studies our in fact comparable. In addition to this, relative values normalized to total protein concentration had no effect on the outcome, probably suggesting that IL-4 and IL-10 are well represented in the total amount of protein exuded in the GCF. Although we found in the literature that men account for higher prevalence compared to women [27], no influence of sex was observed for any of the mediators being analyzed. In other words, sex-specific physiology or steroid hormones would, according to these results, have no effect on the “anti”-mediators being studied.

The different outcomes encountered herein for LL-37 and IL-6 on the one side, and IL-4 and IL-10 on the other, explains the intricate mechanisms behind the disease and its management, where antimicrobial peptides and anti-inflammatory cytokines would not act as a block. Moreover, as recently suggested [43], LL-37 postulates as an optimal candidate for tracking the evolution of the disease at short/middle-term, since unlike anti-inflammatory IL-4 and IL-10, following SRP therapy LL-37 levels decrease to healthy control conditions. Additionally, a positive correlation was found between pain scores and IL-6 (pg/site, $p < 0.001$), but particularly between pain scores and LL-37 ($p < 0.01$) irrespective of normalization method. This would indicate that LL-37 level in GCF could also be taken as a reliable direct pain biomarker in the periodontal disease. Although a previous study reported the relationship between levels of pain intensity and pro-inflammatory cytokines in acute severe pericoronitis [44], to our knowledge this is the first study indicating a positive correlational shift between periodontal pain and crevicular LL-37 following non-surgical conventional treatment. On the other hand, although the correlation between pain and IL-4 or IL-10 levels remained negative ($p < 0.001$), contrary to the distributional shift observed in pain scores, IL-4 and IL-10 were barely affected following SRP therapy.

Finally, it is worth emphasizing the influence of age and oral hygiene habits in the periodontal status and pain perception. Periodontal disease is multifactorial, and the results herein confirm the influence that age [45] and oral habits [46] have on its prevalence, for there is a tendency for the disease to increase in severity along with age. Although the purpose of this work was not to rate the quality of oral hygiene in the patients, we did find that individuals who brushed their teeth less frequently were more common in severe stages of periodontitis as compared to healthy control ($p < 0.05$). In addition, the results showed a positive correlation between the use of a manual instead of an electric toothbrush and periodontal status ($p < 0.01$). However, a limitation of the current study concerns the relatively small sample size. No association was found between the severity of periodontitis and diabetes or smoking habits. We believe that given the low number of subjects with diabetes (n = 1) and smokers (n = 6) included in our study, these limited cases had a little impact on the outcomes obtained. Another limitation is that despite the distribution of individuals in all groups (healthy and different periodontitis stages I-IV) was even, patients were recruited at random. This unfortunately resulted in an older mean age for the periodontal disease groups than for the healthy control ($p < 0.0001$ and $p < 0.001$, respectively).

CONCLUSIONS

The current study highlights the positive correlation between clinical indicators of periodontal status, pain during dental probing and levels of antimicrobial peptide LL-37 and IL-6 in gingival crevicular fluid. On the other hand, the negative correlation against anti-inflammatory cytokines IL-4 and IL-10. A tendency shift is observed for all variables in the former correlation 4–6 weeks following conventional periodontal treatment. We suggest that crevicular IL-6, but particularly LL-37 should stand as a reliable molecular biomarker for therapeutic efficacy (including pain relief) and prognosis in the periodontal disease.

Abbreviations

BL bone loss

BOP bleeding on probing

CAL clinical attachment level

CEJ cement-enamel junction

GCF gingival crevicular fluid

NPRS numeric pain rating scale

PBS phosphate buffer saline

PD probing depth

PI plaque index

SRP scale and root planning

Declarations

Ethics approval and consent to participate

All procedures performed in this study involving human participants were in accordance with the Helsinki Declaration and its later amendments or comparable ethical standards. A protocol was approved by the Ethical Committee of Universidad Rey Juan Carlos (protocol number 2711201916719) and all subjects gave informed written consent after fully explaining the study's aim.

Consent for publication

Not applicable.

Availability of data and materials

All data and materials used are available by the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

DM initiated study concept and design acquisition of data and drafting of the manuscript. MMG performed the molecular assays, contributed to data analysis and participated in the creation of the tables and figures and drafting of the manuscript. LM conducted the data analysis and final review of the manuscript. HH provided his technological expertise for sample size calculation and assisted with study design. GE and LG contributed to drafting and final review of the manuscript. LC supervised the project, provided a factual review and helped edit the manuscript. All authors have read and agreed to the published version of the manuscript.

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Figures

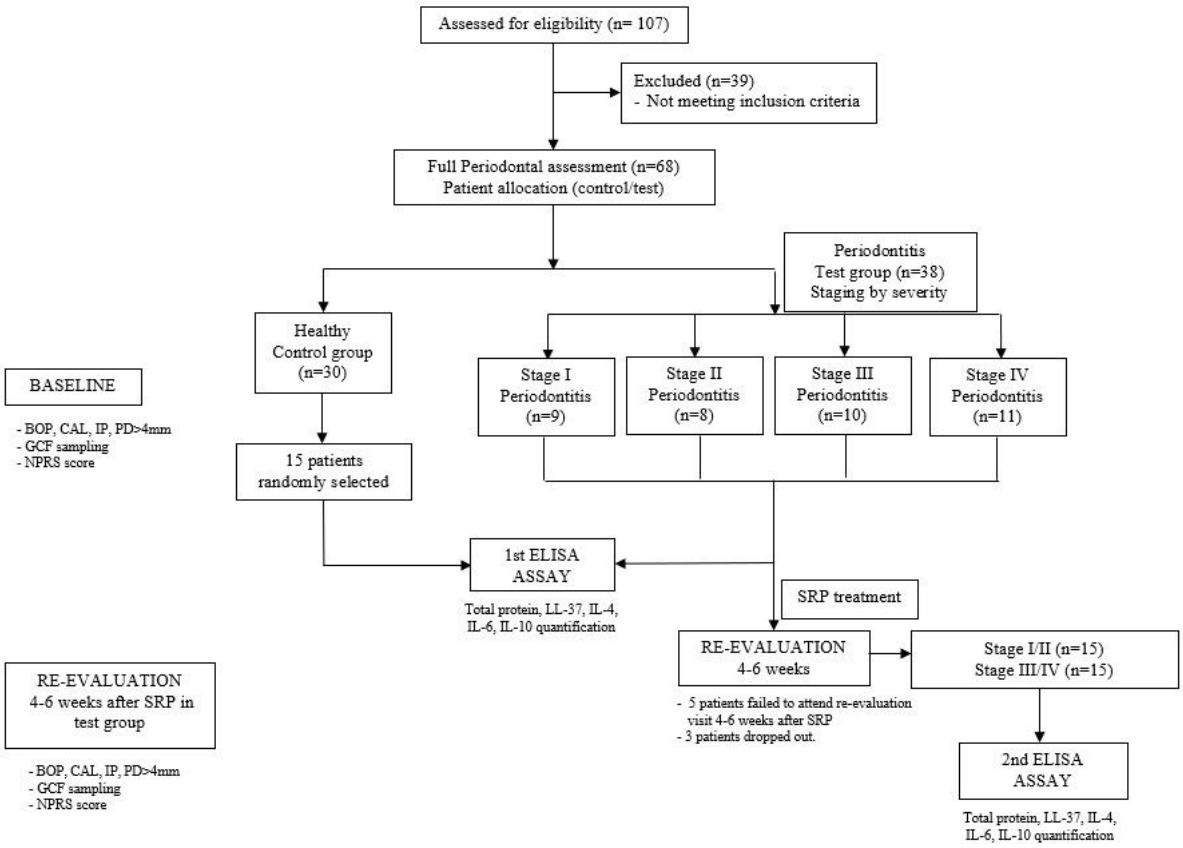

Figure 1

Flow diagram of the selection criteria. n = number of patients.

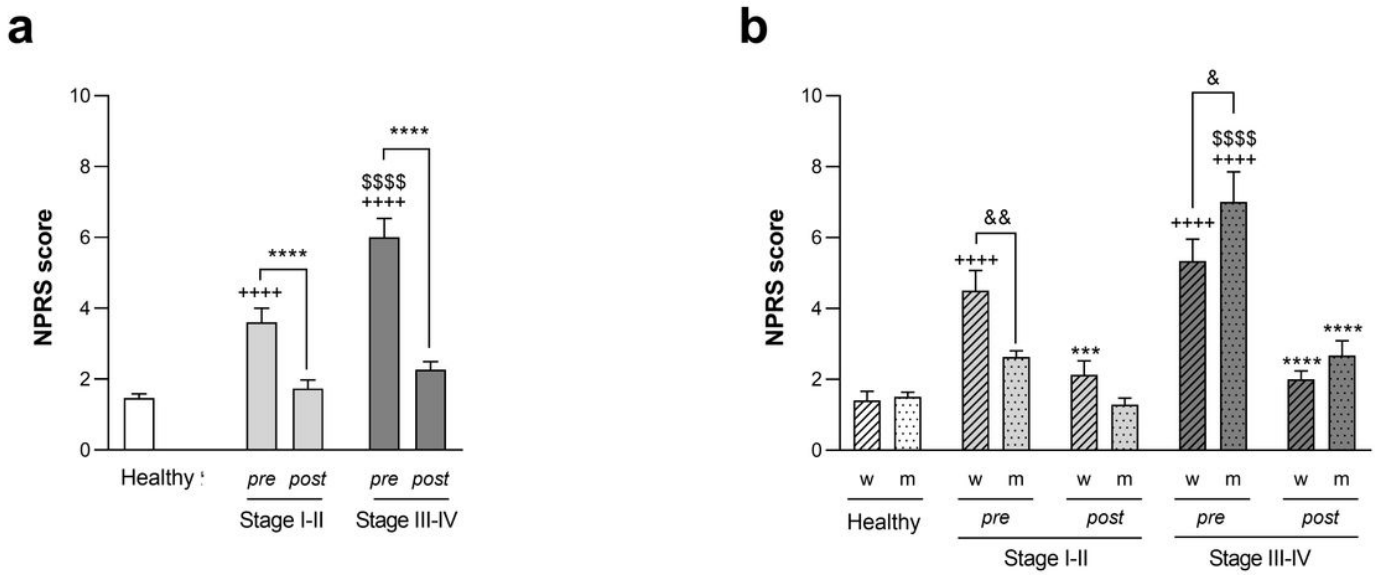

Figure 2

NPRS score for each group before and after SRP therapy. a. Comparative analysis of NPRS score in total sample. $^{\&}$ $p < 0.05$, $^{\&\&}$ $p < 0.01$ women vs. men; two-way ANOVA followed by Sidak's multiple comparisons test. *** $p < 0.001$, **** $p < 0.0001$ pre-SRP vs. post-SRP; $^{++++}$ $p < 0.0001$ vs. healthy control group;

$p < 0.0001$ vs. Periodontitis Stage I-II; two-way ANOVA followed by Tukey's multiple comparisons test. **b.** Comparative analysis in women and men. **** $p < 0.0001$ pre-SRP vs. post-SRP; two-way ANOVA followed by Sidak's multiple comparisons test. $^{++++}$ $p < 0.0001$ vs. healthy control group;

$p < 0.0001$ vs. Periodontitis Stage I-II; two-way ANOVA followed by Tukey's multiple comparisons test. Data are expressed as mean ± SEM. w = women; m = men, pre = pre-SRP, post = post-SRP, NPRS = numeric pain rating scale.

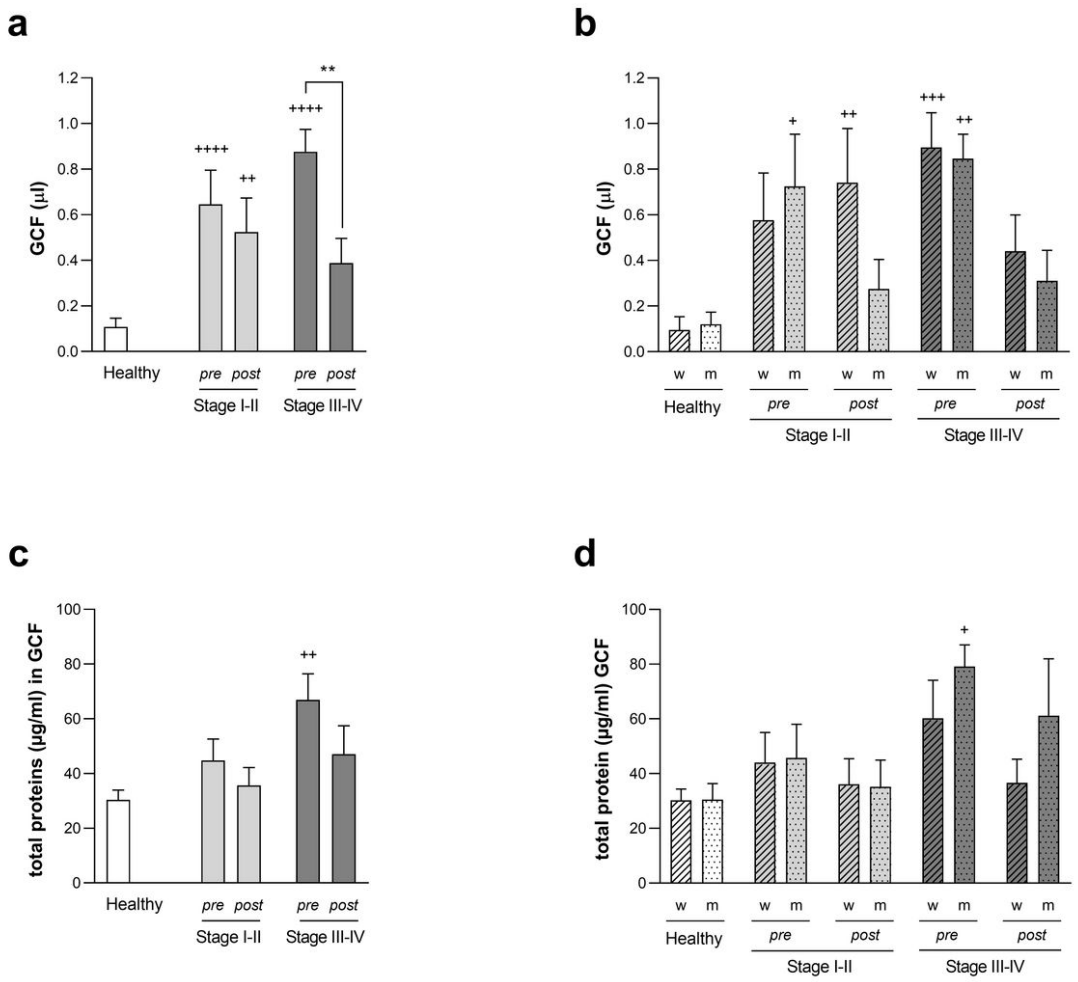

Figure 3

GCF volume (µl) or total protein concentration in GCF (µg/ml) for each group before and after SRP therapy. a. Comparative analysis of GCF volume in total population. **b.** Comparative analysis of GCF volume in women and men. **c.** Comparative analysis of total protein concentration in GCF in total population. **d.** Comparative analysis of total protein concentration in GCF in women and men. Data are expressed as mean ± SEM. *** $p < 0.001$, **** $p < 0.0001$ pre-SRP vs. post-SRP; $^{++++}$ $p < 0.0001$ vs. healthy control group; two-way ANOVA followed by Tukey's multiple comparisons test. w = women, m = men, pre = pre-SRP, post = post-SRP, GCF = gingival crevicular fluid.

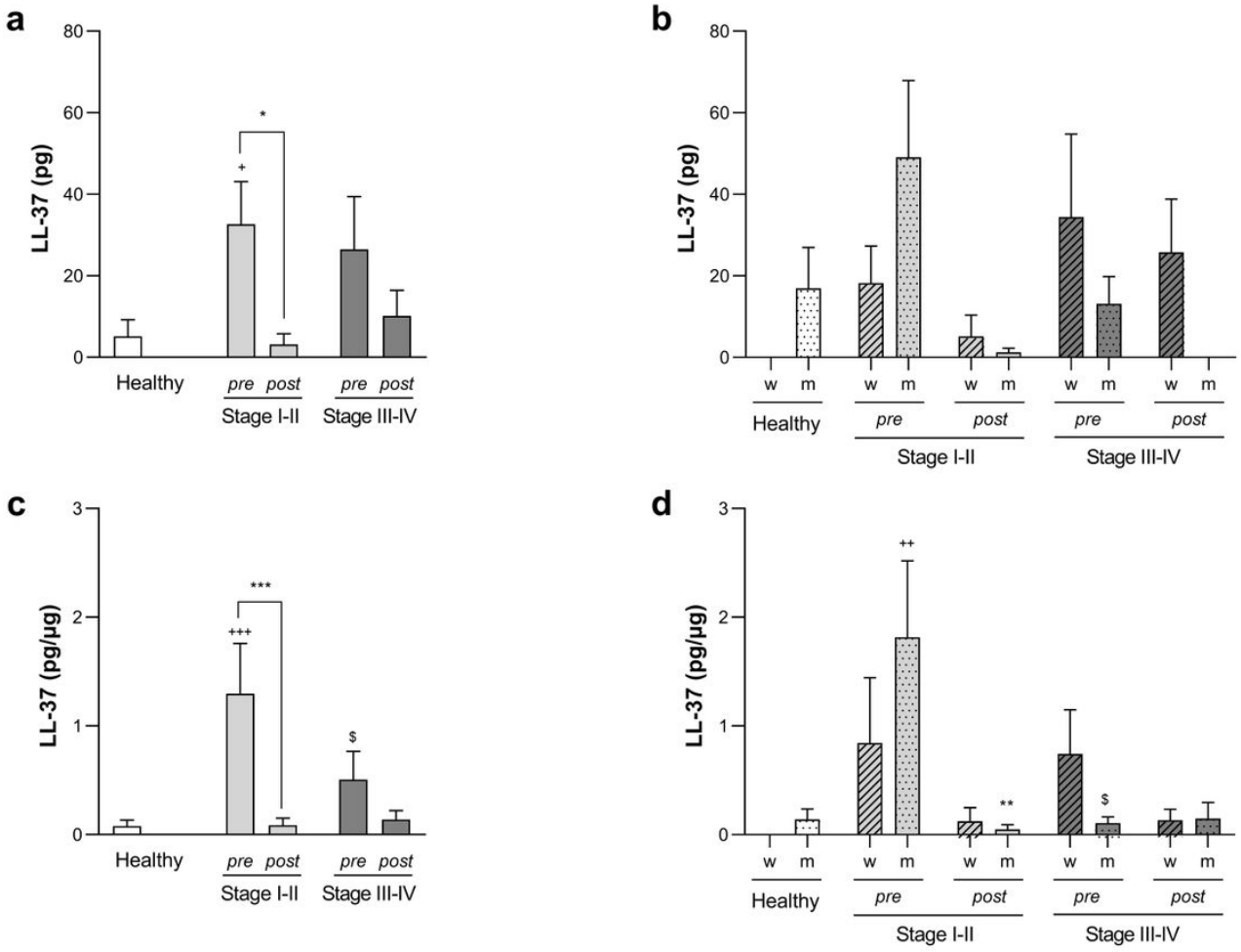

Figure 4

Cathelicidin LL-37 levels in GCF for each group before and after SRP therapy. a. Comparative analysis of LL-37 (pg) in total population. **b.** Comparative analysis of LL-37 (pg) in women and men. **c.** Comparative analysis of LL-37 (pg/µg) in total population. **d.** Comparative analysis of LL-37 (pg/µg) in women and men. Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ pre-SRP vs. post-SRP; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$ vs. healthy control group; $^{\$}$ $p < 0.05$ vs. periodontitis Stage I-II. Two-way ANOVA followed by Tukey's multiple comparisons test. pg = total amount of LL-37 in GCF; pg/µg = concentration of LL-37 to total protein concentration in GCF.

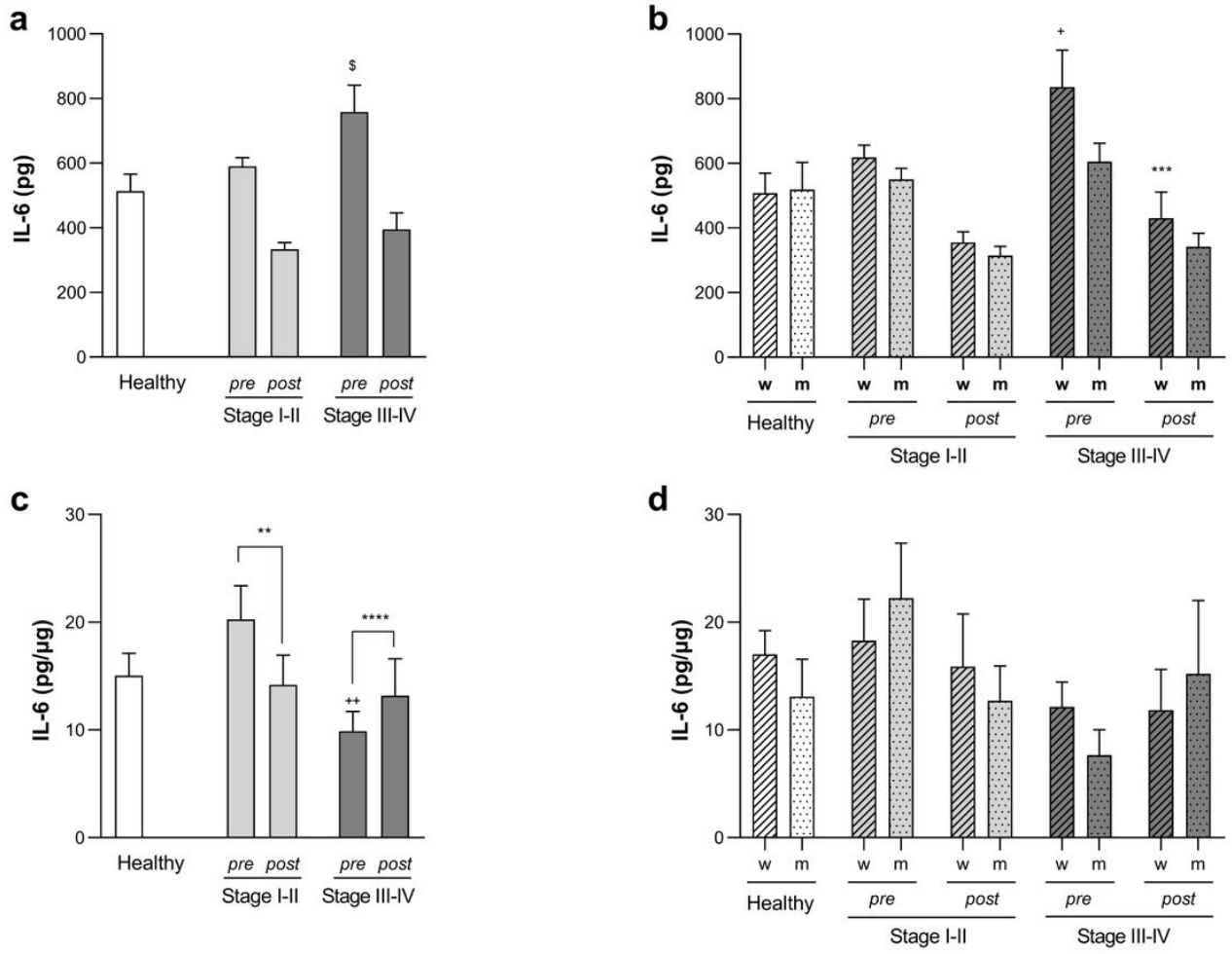

Figure 5

IL-6 levels in GCF for each group before and after SRP therapy. a. Comparative analysis of IL-6 (pg) in total population. **b.** Comparative analysis of IL-6 (pg) in women and men. **c.** Comparative analysis of IL-6 (pg/µg) in total population. **d.** Comparative analysis of IL-6 (pg/µg) in women and men. Data are expressed as mean ± SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ pre-SRP vs. post-SRP; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$ vs. healthy control group; $^{\$}$ $p < 0.05$ vs. periodontitis Stage I-II. Two-way ANOVA followed by Tukey's multiple comparisons test. pg = total amount of IL-6 in GCF; pg/µg = concentration of IL-6 to total protein concentration in GCF.

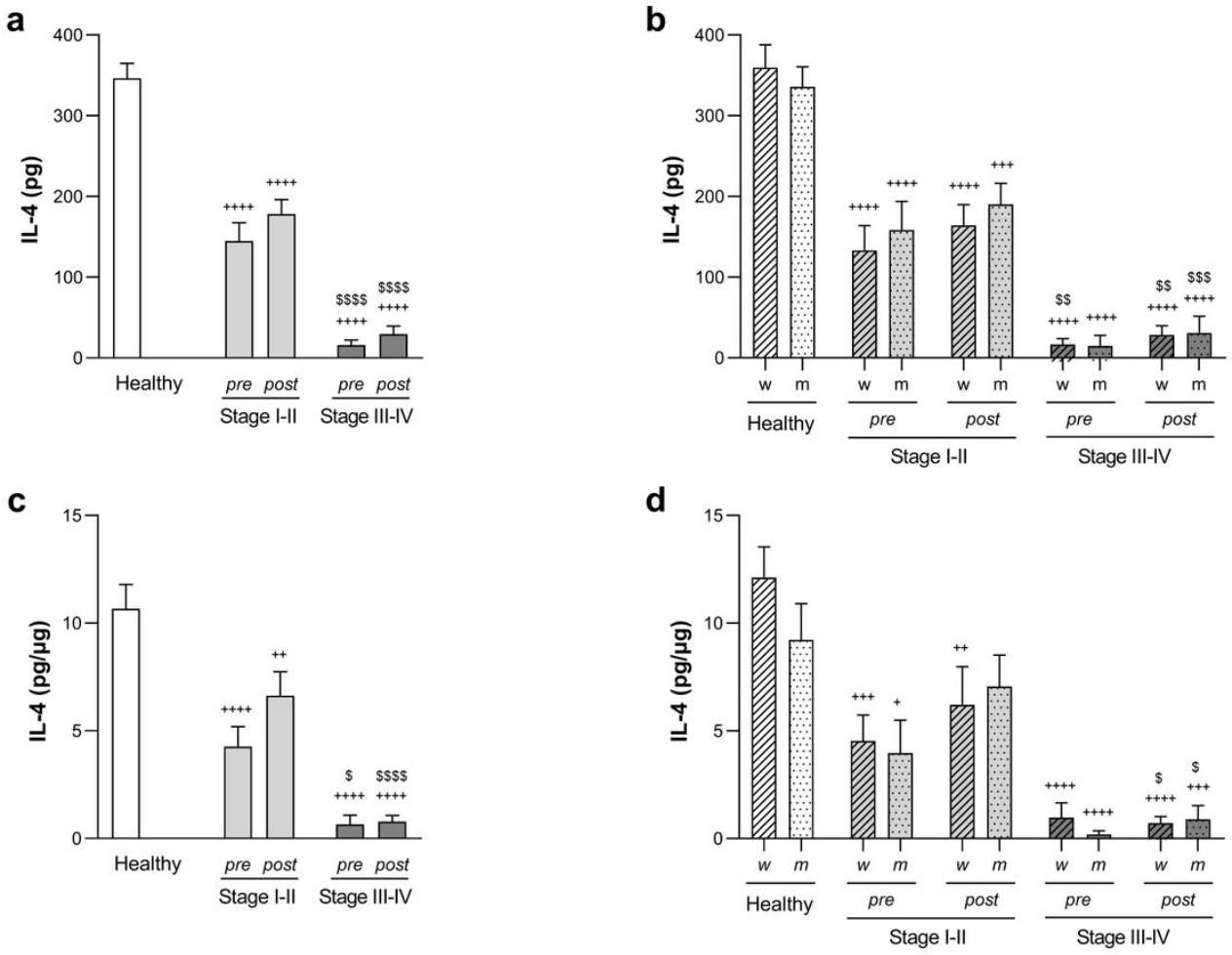

Figure 6

IL-4 levels in GCF for each group before and after SRP therapy. a. Comparative analysis of IL-4 (pg) in total population. **b.** Comparative analysis of IL-4 (pg) in women and men. **c.** Comparative analysis of IL-4 (pg/µg) in total population. **d.** Comparative analysis of IL-4 (pg/µg) in women and men. Data are expressed as mean ± SEM. $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$, $^{++++}$ $p < 0.0001$ vs. healthy control group; $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$,

$p < 0.0001$ vs. periodontitis Stage I-II. Two-way ANOVA followed by Tukey's multiple comparisons test. pg = total amount of IL-4 in GCF; pg/µg = concentration of IL-4 to total protein concentration in GCF.

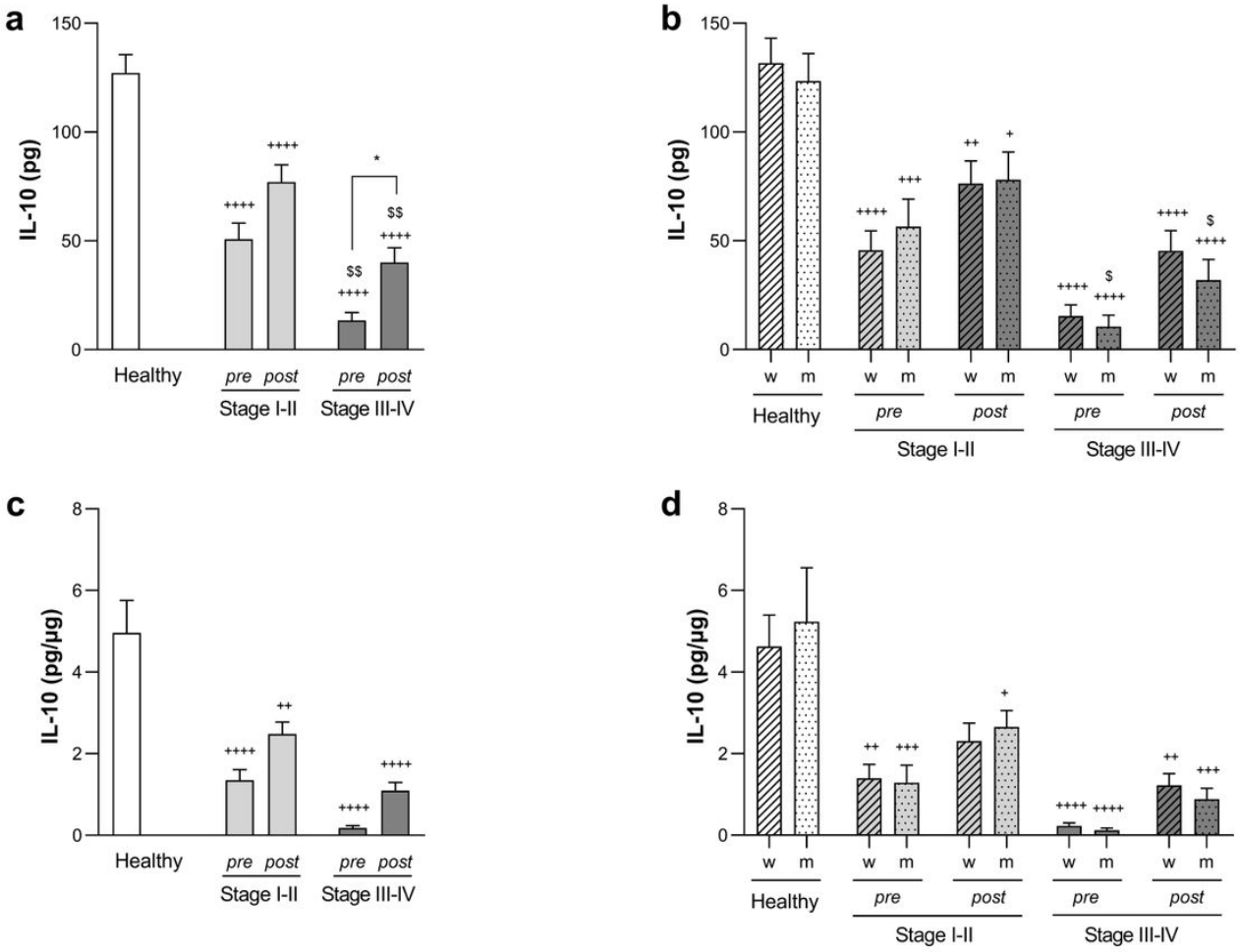

Figure 7

IL-10 levels in GCF for teach group before and after SRP therapy. a. Comparative analysis of IL-10 (pg) in total population. **b.** Comparative analysis of IL-10 (pg) in women and men. **c.** Comparative analysis of IL-10 (pg/µg) in total population. **d.** Comparative analysis of IL-10 (pg/µg) in women and men. Data are expressed as mean ± SEM. * $p < 0.05$ pre-SRP vs. post-SRP; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$, $^{++++}$ $p < 0.0001$ vs. healthy control group; $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$ vs. periodontitis Stage I-II. Two-way ANOVA followed by Tukey's multiple comparisons test. pg = total amount of IL-10 in GCF; pg/µg = concentration of IL-10 to total protein concentration in GCF.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.docx
- Additionalfile2.docx