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Involvement of the endocannabinoid system in current and recurrent periodontitis: A human study

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Abstract

Objective: The aim of the present study was to assess if the endocannabinoid system is involved differently in patients with recurrent and non-recurrent periodontal disease and if in sites that have a predisposition for reactivation, levels of anandamide (AEA) change after periodontal therapy.

Background: Periodontal disease (PD) may be due to a dysregulation of the endocannabinoid system.

Methods: Periodontal patients were recruited, treated for PD and monitored. Gingival samples from these patients with recurrent ($n = 10$) and non-recurrent ($n = 10$) periodontal disease were harvested before and after treatment and compared to those of periodontally healthy ($n = 10$) subjects. Levels of CB1 and CB2, AEA and CBs receptor activation were assessed in healthy and inflamed samples using immunohistochemistry, chromatography and autoradiography. In healed sites, AEA levels were also assessed.

Results: The number of CBs in inflamed sites of recurrent patients was significantly higher than in those with non-recurrent disease and also higher than those in healthy subjects. Inflamed sites of recurrent patients had significantly lower CBs receptor activation than those of healthy subjects. Levels of AEA in inflamed sites of non-recurrent patients were significantly higher than those found both in inflamed recurrent sites and in healthy sites. Otherwise, the amount of AEA in healthy subjects and recurrent inflamed sites was similar. After periodontal therapy, levels of AEA were significantly lower in both periodontal groups. In recurrent sites, they resulted significantly lower than in non-recurrent and even in healthy subjects.

Conclusions: The endocannabinoid system seems involved differently in subjects with recurrent and non-recurrent periodontal disease.

KEYWORDS
anandamide, cannabinoid, CB1, CB2, periodontal disease, periodontitis, receptor activation

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Gaia Pellegrini and Daniela Carmagnola contributed equally to the study.

1 | INTRODUCTION

Periodontal disease (PD) is a chronic and multifactorial condition with a high prevalence.[1,2] The principal etiologic factor is represented by an opportunistic infection of periodontopathogenic bacteria that organize in biofilm adherent to the dental surface.[3–5] Lipopolysaccharides (LPS) of these microorganisms induce the release of inflammatory molecules from neutrophils, macrophages, and other cells of the innate immune response as well as stimulate premature alveolar osteocyte senescence thus contributing to bone loss.[6,7] Clinically, the chronic and progressive infection by bacteria results in the migration of the tissues supporting the teeth in an apical direction, periodontal pocket formation and bleeding on probing of the diseased sites. The extent of tissue destruction following periodontal inflammation is nevertheless limited by the concomitant production of anti-inflammatory mediators and basically, the clinical result of the pathology depends on the equilibrium between pro- and anti-inflammatory mechanisms.[8] Other contributing factors to the onset of periodontal disease are genetic background and environmental factors like smoke, diet, systemic diseases and stress.[9–13]

The goal of the therapy, which consists of the professional removal of local irritants and oral health control, is to arrest the progression of the disease and to maintain healthy periodontal tissues. Usually, once the plaque is removed, resolution of the inflammation is observed.[14] However, in some cases, periodontal therapy does not permanently solve the disease and patients may have a recurrence of the disease despite their rigorous adherence to the periodontal maintenance program and compliance with a satisfactory domestic oral hygienic program.[15] In these patients, the high susceptibility to develop periodontal inflammation may be due to a dysregulation of the immune system.

The endocannabinoid system (ES) plays a role in the regulation of immune responses even in the case of periodontal disease.[16–18] It includes some lipidic mediators deriving from arachidonic acid (anandamide AEA; 2-Arachidonoylglycerol 2-AG), their receptors and the enzymes responsible for their synthesis and degradation. CBs receptors, termed CB1 and CB2, belong to the family of G protein-coupled receptors (GPCR) and exist in an inactive and active functional state. In the inactive state, the receptor couples to a trimeric (α, β, γ) G-protein bound to GDP. In the active state, the receptor binding to the G-protein reduces its affinity for GDP. Therefore, GDP detaches and the G-protein binds to GTP. Assessment of the receptor activation allows the studying of the ES's functional activity. At the basal level, only a part of these receptors has the active conformation and the active/inactive binding states are in equilibrium. Once the receptor binds to the agonist, its active conformation is promoted, the signaling pathway is activated, and the equilibrium of the tissue shifts towards the active state. Agonist molecules have a low affinity for receptors uncoupled to G-proteins and a high affinity for coupled receptors.[19,20] Otherwise, antagonists have the same affinity for both coupled and uncoupled receptors and do not affect the balance of the activity state.[20]

When infection or LPS-mediated inflammation occurs, the immune and resident cells respond to these stimuli even through the involvement of the ES by secreting AEA and 2-AG (agonists of CBs) and upregulating the CBs. Once activated, these receptors modulate the release of pro-inflammatory cytokines.[21–23] In patients with periodontal disease, the involvement of the endocannabinoid system showed anomalies,[22,24] and to diminish the inflammatory response it has been proposed the local injection of AEA.[25] However, literature reports contrasting (pro- and anti-inflammatory) effects of AEA in IL-1β stimulated periodontal fibroblasts raising the necessity to have more precise information regarding the involvement of ES, the identification of anomalous CB1-CB2 expression/activation as well as the AEA production in periodontitis.[21,26] In particular, the comprehension of differences in pathogenetic mechanisms between subjects that respond well to the therapy and those that have a recurrence of the disease may help to design a personalized preventive approach to the recurrent tissue damage and more efficacious therapy.

The present study aimed to assess if in patients with the recurrence of periodontal disease the endocannabinoid system activates differently than in healthy subjects and patients with non-recurrent PD. Furthermore, we investigated if, in sites that have a predisposition for reactivation, AEA levels after periodontal therapy are anomalous.

2 | MATERIALS AND METHODS

2.1 | Study design

The present observational study was performed in two private practices by periodontal specialists.

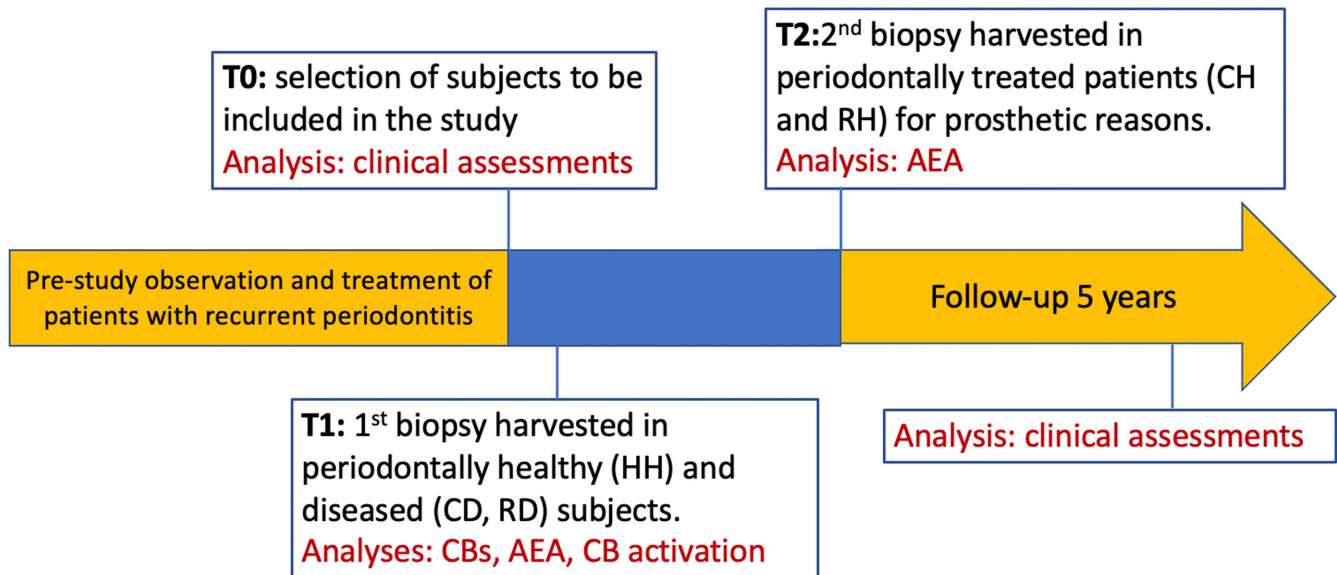Three kinds of subjects were included at T0:

1. Healthy subjects.
2. Patients with recurrent periodontal disease.
3. Patients with non-recurrent periodontitis.

The expression of CB1 and CB2 receptors, the activation of CBs receptors, and levels of AEA were assessed in the healthy gingival tissue of healthy subjects and in inflamed gingival tissue of patients with recurrent and non-recurrent periodontal disease (T1). Furthermore, levels of AEA were also assessed in healed gingival tissue of the patients with periodontal disease (recurrent and non-recurrent) after non-surgical periodontal therapy (T2).

2.2 | Study population

The study design is reported in Figure 1.

At the first visit (*T0*), all participants, underwent examination, charting and ultrasonic supragingival calculus removal and were

T0: selection of subjects to be included in the study
Analysis: clinical assessments

T2: 2nd biopsy harvested in periodontally treated patients (CH and RH) for prosthetic reasons.
Analysis: AEA

Pre-study observation and treatment of patients with recurrent periodontitis

Follow-up 5 years

T1: 1st biopsy harvested in periodontally healthy (HH) and diseased (CD, RD) subjects.
Analyses: CBs, AEA, CB activation

Analysis: clinical assessments

FIGURE 1 Timeline of the study. At T0 participants of the study were selected. Biopsies were harvested at T1 in healthy subjects (HH) during tooth extraction and non-surgical periodontal therapy in patients with recurrent (RD) and non-recurrent (CD) periodontal disease. At T2, biopsies were collected in healed sites of subjects with recurrent (RH) and non-recurrent (CH) periodontal disease during resective surgery. Levels of Anandamide (AEA) and the amount of CBs receptors were assessed.

enrolled according to the inclusion/exclusion criteria and characteristics that are reported below.

Informed consent was obtained from all participating subjects. The principles outlined in the Declaration of Helsinki on experimentation involving human subjects were followed. The present study was approved by the Ethical Committee of "Università degli Studi di Milano" (protocol number 36/11 of 20/12/2011).

Exclusion criteria were the presence of cardiovascular disease, bone metabolic disorders, severe diabetes or anemia, pregnancy or lactation, therapy with immunosuppressant and anti-epileptic drugs, bisphosphonates, opiates, anti-inflammatory drugs, antibiotics in the last 2 months, radiotherapy or chemotherapy, tobacco and cannabis smoking, having less than 20 teeth, the presence of implants. Patients included in the study fulfilled specific inclusion criteria:

1. Healthy subjects: ($n = 10$) had no interdental loss of clinical attachment level, no tooth loss due to periodontitis, no radiographic sign of bone loss, FMPS (Full Mouth Plaque Score) and FMBS (Full Mouth Bleeding Score) <5% and scheduled for third molar extraction;

2. Patients with recurrent periodontal disease ($n = 10$) were subjects with a past diagnosis of "refractory" periodontitis, already treated with scaling, root planning and periodontal surgery when needed, who satisfactorily comply with recommended oral hygiene procedures and follow a rigorous program of periodontal maintenance, but who did not definitely respond to periodontal therapy and exhibited additional clinical attachment loss. Such patients had to have at least 2 "active" sites at the time of recruitment, that is sites bleeding on probing and with pocket probing depth (PPD) ≥5 mm, together with a FMPS <25% and good compliance. According to

the new classification, they would be classified as stage III and grade C, but considering the time when the study was designed, they were considered "refractory" according to the definition of the American Academy of Periodontology.[15,27] After periodontal treatment, these patients were followed up for 5 years and reported at least an episode of periodontal disease reactivation.

3. Patients with periodontal non-recurrent ($n = 10$) periodontitis. According to the new classification, they would be classified as stage II and III and grade B, but considering the time when the study was designed, they were considered chronic patients according to the definition of Armitage (1999). Periodontitis was defined as the presence of at least four active sites (i.e. a periodontal pocket ≥5 mm and bleeding on probing -BOP-), the presence of calculus and FMPS and FMBS >25%.[27,28] Patients allocated in this group had never been treated for periodontal disease before their inclusion into the study. Furthermore, after treatment (T1) they reached a complete resolution of periodontal disease (no further clinical radiographic loss of bone and interdental clinical attachment level or teeth due to periodontitis), followed a satisfactory program of professional and domiciliary maintenance of hygiene for the following 5 years.

At the level of the experimental site, the features, inclusion criteria and codification are reported below.

In healthy subjects, the experimental site was the gingiva adjacent to the third molar to be extracted. To be included in the protocol, the site was free from signs of inflammation, bone resorption, CAL loss, plaque and debris accumulation (*HH: healthy site in healthy subject*).

In patients with recurrent periodontitis, two experimental sites were defined:

1. *RD* (*inflamed site in patients affected by recurrent disease*): Active site at T1 with PPD >6 mm and bleeding on probing, harvested during non-surgical therapeutical procedures;

2. *RH* (*healed site in patients affected by recurrent disease*): A healed site within the same sextant as the RD, that at the time of harvesting (T2: 3–6 months after non-surgical therapy) resulted free from signs of inflammation, plaque, debris accumulation and with PD <4 mm and suitable for surgery for prosthetic reasons.

In patients with non-recurrent periodontitis, two experimental sites were defined:

1. *CD* (*inflamed site in patients affected by non-recurrent disease*): Active site at T1 with PPD >5 mm and bleeding on probing, harvested during non-surgical therapeutical procedures;

2. *CH* (*healed site in patients affected by non-recurrent disease*): A healed site within the same sextant as the CD, that at the time of harvesting (T2: 3–6 months after non-surgical therapy) resulted free from signs of inflammation, plaque, debris accumulation and with PD <4 mm and suitable for surgery for prosthetic reasons.

In the group of healthy subjects, during the following appointment (T1) the third molar extraction was performed under local anesthesia and then a biopsy of gingival tissue was obtained from the region adjacent to the third molar (HH).

In the groups of patients with recurrent and non-recurrent periodontitis during the following appointment (T1) non-surgical periodontal therapy was performed. Briefly, all pockets were cleaned under local anesthesia using ultrasonic scalers and hand instrumentation (Gracey standard and mini five curettes), and the roots were planned. The treatment was performed by the same operator following a quadrant-by-quadrant approach. At this time, one biopsy of the experimental inflamed site, usually at the palatal/lingual part of the interdental papilla, was performed both in recurrent (RD) and non-recurrent (CD) PD patients.

At 3–6 months after non-surgical therapy, the charting was performed to re-evaluate the status of periodontal health and resective surgery was planned for prosthetic reasons.

During the appointment for the resective surgery (*T2*), after local anesthesia the operator selected the site adjacent to the one where the first biopsy was collected at T1. The site needed to be free from any sign of inflammation, plaque, debris accumulation and with PD <4. During the surgery (T2), the biopsy of the experimental healed sites was performed (CH and RH).

Therefore, at T1 we obtained the following biopsies (about 5 mm^3):

1. One healthy site from healthy subjects (HH);
2. One diseased site from non-recurrent and one from recurrent patients (RD, CD).

Each of these samples was cut into three parts and processed separately for the quantification respectively of CB1-CB2, G-proteins for CB activation and AEA. The first (for CBs) was immersion-fixed in formalin, the second (for G-proteins) part was cryofixed in OCT (Optimum Cutting Temperature) and the third one (for AEA) was frozen.

At T2, one biopsy (about 2 mm^3) from the healed site of non-recurrent and recurrent PD patients (RH, CH) was harvested, frozen and only processed for AEA quantification.

2.3 | Histological and immunohistochemical assessment of CB1 and CB2

The first part was immersed and fixed in 4% formalin/0.1 mol/L phosphate-buffered saline (pH 7.4) for 48 h at room temperature and then processed for paraffin embedding. The samples were dehydrated in increasing concentrations of ethanol (50%, 70%, 80%, 90%, 96%, 100%), placed in xylol for 12 h and then embedded in paraffin. Serial longitudinal sections of about 4–5 μm were obtained, mounted on 3-Amino-propyl-trietoxi-xilane coated slides and then hydrated in decreasing concentrations of xylol and ethanol (from 100% to 50%) and finally, immersed in distilled water. For morphological assessment of the tissue, some slides of each biopsy were stained with Mayer hematoxylin/eosin and then observed and photomicrographed under a Nikon light microscope equipped with a calibrated digital camera (Eclipse E600, Nikon) (DXM1200, Nikon). The remaining slices were processed for immunohistochemistry. Antigen retrieval was obtained through Proteinase K solution at 37°C in a humidified chamber for 20 min. The slices were incubated for 60 min at room temperature with CB1 and CB2 primary antibodies (1:200) (Biotechnology). Finally, the sections were washed with a PBS solution three times for 6 min, treated with a polymeric labelling system kit (UltraVision Quanto Large Volume Detection System HRP Polymer) and revealed with diaminobenzidine. The sections were counterstained with Mayer's hematoxylin, observed and photomicrographed with a Nikon light microscope equipped with a calibrated digital camera (Eclipse E600, Nikon) (DXM1200, Nikon). Three sections were obtained from each sample. Each section was photomicrographed 10 times at a 200x magnification and on each picture the presence and distribution of CB1 and CB2 in the gingival tissue were evaluated separately for epithelium and connective tissue, using an image processing software (PhotoShop, Ps5). A specific color range was set to evaluate the percentage of marked tissue over each area, calculated as the fraction of the marked pixels over the total pixels. The color range excluded non-specific sample marking.[29]

2.4 | Autoradiography for G-protein detection

Concerning the second bioptical part, G-proteins were analyzed to evaluate the activation CBs. Agonist-stimulated [35S]GTPγS binding in autoradiography CP-55940-stimulated [35S]GTPγS binding was determined as described previously.[30] Briefly, samples were cryofixed in OCT (Optimum Cutting Temperature) the gel media used for

frozen sections, and sectioned on a cryostat, then the slides were incubated in assay buffer (50 mM Tris–HCl, 3 mM MgCl2, 0.2 mM EGTA, 100 mM NaCl, 0.1% BSA, pH 7.4) at 25°C for 10 min and then in 3 mM GDP in assay buffer at 25°C for 15 min. The slides were transferred into an assay buffer containing 3 mM GDP and 0.04 nM [35 S]GTPγS (Perkin Elmer Life Sciences) with (stimulated) or without (basal) 5 μM CP-55940 (Tocris) and incubated at 25°C for 2 h. They were then rinsed twice in cold 50 mM Tris buffer and once in deionized water, dried and exposed to Kodak Biomax MR films (Perkin Elmer Life Sciences) for 48 h.

For image analysis, the intensity of the autoradiographic film was assessed by measuring the grey levels with a dual scanner Artixscan 1800F connected to a PC running Image-Pro Plus v.5.0 software (Media Cybernetics Inc.). The grey level of densitometric measurements calculated after subtracting the background density was established within the linear range determined using 35 S standards made in the laboratory.

2.5 | Chromatography for assessment of AEA levels

On the third biopsy, the levels of AEA were measured as reported below.

2.5.1 | Extraction and derivatization procedures

Each sample of the gingiva, weighted at room temperature, was collected into monovettes in aliquots of 10 mg of the tissue and frozen at −70°C. Cut while still frozen in 1.5-mL polypropylene tubes, the tissues were homogenized using a suitable tissue grinder in an ice-cold Tris buffer pH 7 (20 mcL). These samples were spiked with d8-AEA (5 mcL of a 1 mcg/mL solution of d8-AEA in ethanol) at a final concentration of 5 ng. After conditioning for 10 min, for each sample was performed solvent extraction with toluene (200 mcL) for 15 min on a Rotary-agitator (Falc). After centrifugation with Fisher Bioblock Scientific Sigma (23 548 g force × min, 10 min, 10°C), the clear, color-less supernatant toluene phase was transferred into a glass vial and toluene was evaporated to dryness under a gentle nitrogen stream. BSTFA N,O-Bis(trimethylsilyl)trifluoroacetamide (50 mcL) was added to the residue and was incubated at 25°C for 30 min for analyte sialylation. Then, the solvent was evaporated and the residue was dissolved in hexane (50 mcL).

2.6 | GC–MS/MS analysis

Analyses were performed in positive-ion chemical ionization (PCI) mode on a triple-stage quadrupole mass spectrometer Agilent 7000B (Agilent Technologies Santa Clara) directly interfaced with a 7890A series gas chromatograph equipped with an autosampler. Chromatographic separation took place on a column Phenomenex Zebron for "semi volatiles" fused silica column 20 m × 0.18 mm i.d., 0.18 mcm film thickness. In quantitative analyses, the electron multiplier voltage was set to 2800 V. Methane (530 Pa) and argon (0.2 Pa pressure in the collision cell) in GC–MS/MS were used as reagents and collision gases, respectively. Aliquots (1 mcL) were injected in the splitless mode by using an Agilent injector, at the temperature of 250°C, carrier gas, and helium at a constant flow rate of 1.0 mL/min. The following oven temperature program was used: 100°C for 1 min, increased to 300 at 25°C/min kept at 300 for 3 min Electron energy was set to 200 eV. Quantitative measurements were conducted on the AEA–TMS derivatives by selected reaction monitoring (SRM) the transition 330.0, 133.8 m/z for AEA and 338 337 m/z for D8-AEA, generated by collision-induced dissociation (CID) of the precursor ion 420 m/z of AEA-TMS and 428 m/z of D8-AEA-TMS.

The limit of detection (LOD) of this assay was 0.02 ng/mg, determined as the quantity of standard injected that produced a peak greater than three times the standard deviation of background noise.

Calibration curves for quantitative analyses were constructed by adding increasing amounts (0–0, 1-0, 2–0, 5–1-2, 5–5–10-25 ng) of unlabeled AEA. Regression analysis indicated that the plots for AEA were linear over the range of 0–25 ng ($r^2 = .9995$) with the regression equation $y = 0.005 + 0.61\, x$.

2.7 | Statistical analysis

From the analysis, the mean and SD of the following data were obtained:

1. Amount of CB1, CB2, CB1 + CB2 in the epithelium and connective tissue, and CBs total content (CB1 + CB2 in epithelium + connective tissue) expressed as % of the tissue (for HH, RD and CD);
2. Amount of G-protein activated as % of CBs net stimulation (for HH, RD and CD);
3. Amount of AEA (pg/mg) in the tissue (for all groups);

To describe the anatomical distribution of CBs within the tissue during the three clinical situations at T1 and to assess if inflamed tissues express them abnormally, the following investigations were done.

In each group of subjects (HH, RD, CD) the number of receptors (CB1, CB2 and CB1 + CB2) found in the epithelium was compared to that found in the connective tissue using the Wilcoxon Signed-Ranks Test. The inter-disease-group (healthy vs recurrent vs nonrecurrent) differences in levels of CBs total content were analyzed by the Kruskall-Wallis Test. In case of emerging significance between the overall three disease groups, the Mann–Whitney Test was used to compare pairs of groups.

To assess the between-group differences for G protein activation the Kruskall-Wallis Test was applied.

To assess if the inflammation may increase tissue levels of AEA, the Kruskall-Wallis Test was used to compare data of HH vs RD and

CD. In case of emerging significance between the overall three disease groups, the Mann–Whitney Test was used to compare pairs of groups.

To evaluate if, in consequence of periodontal therapy and resolution of the inflammatory disease, sites with non-recurrent and recurrent periodontitis return to basal levels of AEA similar to those of HH, the Kruskall-Wallis Test was firstly used to compare data of HH, RH and CH. Then in case of emerging significance, the Mann–Whitney Test was used to compare pairs of groups.

For significance, p was set at $< .05$.

3 | RESULTS

A total of 30 participants were enrolled in the study (10 for each disease group) from 2012 to 2015 and followed up until 2021. Data on the study population are reported in Table 1.

3.1 | Tissue morphology

At histological assessment, the gingiva of healthy or healed sites of all groups (HH, RH and CH) (n: 10 per group) appeared free of inflammatory infiltrate. At higher magnification, the epithelium had a normal architecture with clearly identifiable basal, spinous, granular and cornified layers. The connective tissue showed organized collagen fibers with a regular distribution of the blood vessels and the absence of inflammatory cells.

In samples from inflamed sites of both recurrent and non-recurrent PD patients, morphological alterations were evident. The epithelium was thickened with increased epithelial crest projections in the connective tissue. The connective tissue appeared loose. At higher magnification, the junctional epithelium was not well defined, with large intercellular spaces. A conspicuous presence of inflammatory cells and enlarged blood vessels was observed. No differences in the microstructure and organization of the inflamed gingival tissue appeared between groups (Figures 2 and 3).

TABLE 1 Demographic and periodontal clinical data of healthy subjects (HH) and patients with recurrent (RD) and non-recurrent (CD) and periodontal disease at T0.

	HH	RD	CD
Male/Female	4/6	6/4	5/5
Age (mean, range)	45.1 (34–55)	40.9 (31–50)	51.2 (36–68)
PPD >6 mm	0%	1.4% + 0.5%	5.6% + 1.3%
PPD 4–6 mm	0%	3.2% + 1.8%	13.1% + 5.3%
FMPS	2% + 0.4%	7.2% + 1.5%	28.3% + 2.4%
FMBS	0.5% + 0.1%	16.6% + 2.8%	27.6% + 1.7%

Note: The percentage of sites with periodontal pocket depth (PPD) = /> 4 mm, Full Mouth Plaque Score (FMPS) and Full Mouth Bleeding Score (FMBS) were assessed.

3.2 | Assessment of CB1 and CB2

At the observation, CB1 and CB2 receptors of subjects with periodontal recurrent and non-recurrent disease appeared localized mainly in the superficial layers of the epithelium (granular and cornified layers). No difference in the distribution of the receptors was observed between epithelial layers and between epithelium and connective tissue in healthy subjects.

Descriptive data of CB1 and CB2 amount in the epithelium, connective tissue and total content of the experimental sites HH, CD and RD (n: 10 per group) are reported in Table 2.

Inferential statistic. At the within-group analysis, it resulted that:

1. In both RD and CD, epithelium showed significantly higher levels of CBs receptors (CB1+CB2) than connective tissue ($p < .05$);
2. In RD sites, levels of both CB1 and CB2 were higher in the epithelium tissue (respectively 12.77 and 13.04 pg/mg) than in the connective tissue (4.17 and 3.82 pg/mg) ($p < .05$);
3. In CD sites, the epithelium amount of CB2 (3.62 pg/mg) resulted significantly higher than the amount of CB1 (2.56 pg/mg). CB2 in the epithelium (3.62 pg/mg) was significantly higher than its level in the connective tissue (2.14 pg/mg) ($p < .05$).

Since a homogenous trend of CB1 and CB2 expression in epithelium and connective tissue was found in all groups, pool data (total CBs content) were used. At the Kruskall-Wallis test, data resulted significantly different ($p < .0001$). In particular, the number of receptors in inflamed sites of patients with the recurrent disease was significantly higher than in those with non-recurrent disease and also higher than those in healthy subjects (RD > CD and RD > HH, the Mann–Whitney Test $p < .001$). CBs amount in CD sites was also higher than in HH sites (the Mann–Whitney Test $p < .001$).

3.3 | Assessment of G-protein activation

Data on CP-55940-stimulated [35S]GTPγS binding for HH, RD and CD (n: 10 per group) are reported in Figure 4.

At the Kruskal-Wallis test, differences between groups resulted significantly different ($p < .0001$). At the analysis, inflamed sites of patients with non-recurrent periodontal disease had significantly lower activation of G-protein than healthy subjects (CD < HH, Mann–Whitney Test $p < .05$). Otherwise, inflamed sites of recurrent PD patients did not show any difference in both groups.

3.4 | Levels of anandamide

Levels of anandamide detected at T1 (HH, RD, CD) (n: 10 per group) and T2 (RH and CH) (n: 10 per group) in the gingival tissue are reported in Figure 5.

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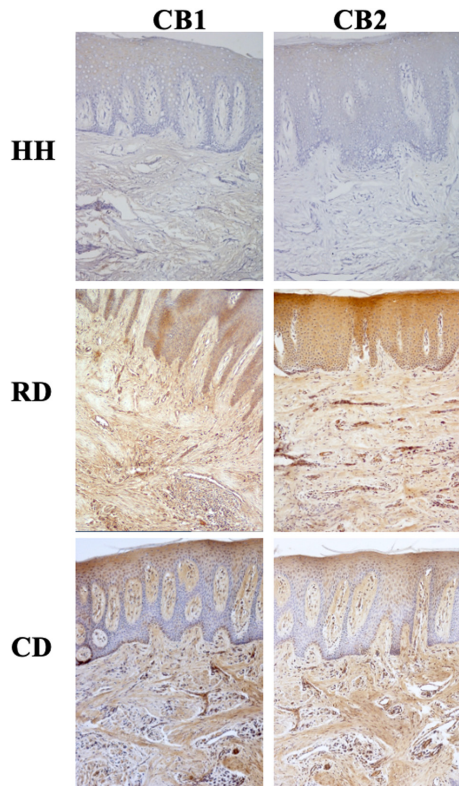
FIGURE 2 Photomicrographs of samples harvested at T1 from healthy subjects (HH) and patients with recurrent (RD) and non-recurrent (CD) periodontal disease after CB1 and CB2 immunostaining. The immunostaining resulted in more evidence in the epithelium and, in particular, in its superficial layers. In inflamed sites, intense inflammatory infiltrates and numerous enlarged vessels appeared. CB1 and CB2 immunostaining. Total magnification 10×.
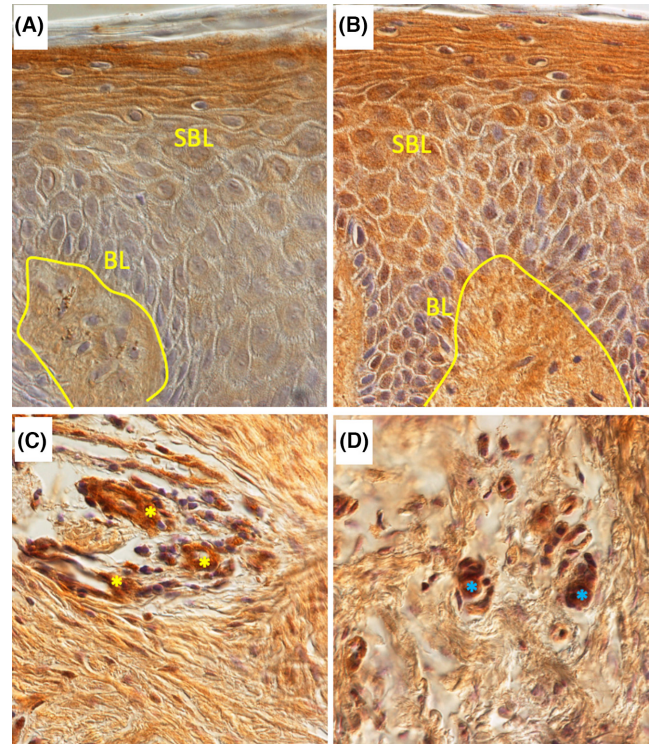
FIGURE 3 Photomicrographs of samples harvested at T1 from patients with recurrent periodontitis and immunostained for CB1 (A) and CB2 (B–D) receptors. In the epithelium, suprabasal (SBL) epithelial layers (granular and cornified layers) appeared more intensely stained than basal (BL) and spinous layers for both CB1 and CB2. In the connective tissue (C and D), immune cells including macrophage-like cells (yellow asterisks) and neutrophil-like cells (blue asterisks) appeared to express CB2 receptors. The basal membrane is indicated with the yellow line. Total magnification 60×.

TABLE 2 Mean values and standard deviation of CB1 and CB2 receptors in the epithelium and connective tissue of healthy subjects (HH) and patients with recurrent (RD) versus non-recurrent (CD) periodontal disease at T1.

	Epithelium		Connective tissue		
	CB1	CB2	CB1	CB2	TOT
HH	0.06 (0.02)	0.03 (0.02)	0.14 (0.09)	0.09 (0.05)	0.32 (0.19)
RD	12.77 (6.09)	13.04 (4.27)	4.17 (0.99)	3.82 (1.07)	33.80 (12.41)
CD	2.56 (0.47)	3.62 (1.14)	2.14 (0.47)	1.99 (0.49)	10.31 (2.56)

Note: The CBs total content (CB1 + CB2 in epithelium + connective tissue) is also reported.

3.4.1 | Inferential statistics

At T1, emerged that AEA in CD sites was double that in HH sites. In recurrent, AEA levels were comparable to healthy subjects. Differences between these groups resulted in significant (HH vs RD vs CD) ($p < .001$). In particular, levels of AEA in inflamed sites of patients with non-recurrent periodontal disease were significantly higher than those found both in inflamed sites of patients with recurrent disease (CD > RD, $p < .005$) and in healthy subjects (CD > HH, $p < .0005$). Otherwise, the AEA amount in HH and RD resulted similarly.

After periodontal non-surgical therapy and resolution of tissue inflammation (T2), levels of AEA reduced significantly in both periodontal patients (intragroup assessment, CD > CH and RD > RH, $p < .05$, Wilcoxon Signed-Rank Test). At the comparison of levels of this protein in healthy and healed sites significantly differences resulted (HH vs. RH vs. CH, intergroup analysis, $p < .05$). Healed sites of patients with non-recurrent disease had a similar amount of AEA than healthy subjects (CH = HH). On the contrary, healed sites of patients with recurrent disease (RH) had a significantly lower amount of AEA in the gingiva than both HH and CH ($p < .05$).
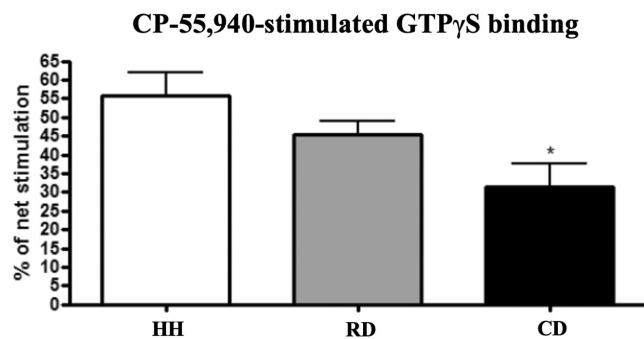
CP-55,940-stimulated GTPγS binding

FIGURE 4 Percentage of active CBs receptors in healthy subjects (HH), and patients with recurrent (RD) and non-recurrent (CD) periodontal disease. Kruskall-Wallis Test *$p < .05$.
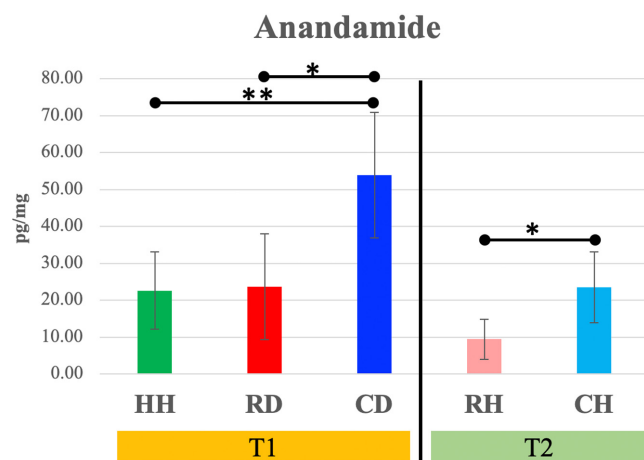
Anandamide

FIGURE 5 Data of Anandamide amount at T1 in healthy subjects (HH) and in inflamed sites of patients with recurrent (RD) and non-recurrent (CD) periodontal disease. After periodontal non-surgical therapy (at T2), the AEA amount was also evaluated in healed sites in both recurrent (RH) and non-recurrent (CH) periodontal patients. Mann–Whitney Test, *$p < .005$; **$p < .0005$.

4 | DISCUSSION

The present manuscript aimed to investigate if a different involvement of the endocannabinoid system may occur in subjects with current and recurrent periodontal disease. Healthy subjects were chosen as the negative control.

First, the distribution and the number of CBs in the gingival epithelium and connective tissue were assessed in inflamed and healthy sites, in which we also assessed the functional activation of the CB receptors.

The histomorphometry revealed that in healthy subjects CB1 and CB2 receptors were equally expressed without differences between epithelium and connective tissue. When inflammation occurs, both in recurrent and non-recurrent patients, expression of these receptors resulted upregulated, in particular in the superficial layers (granular and cornified layers) of the epithelium that showed significantly higher levels of receptors compared to connective tissue. These data confirm the involvement of CB1 and CB2, and of the epithelium

associated with periodontal inflammation, in line with the results of previous research.[22,24] Studies observed that cannabinoid receptors seem to play a role in the promotion of periodontal tissue healing by the improvement of fibroblast adhesion and migration, and by enhancing the osteo/dentinogenic differentiation of periodontal ligament stem cells even during tissue inflammation.[17,24,31,32] According to our results, inflammation does not seem to impact predominantly on one of the two receptors. Literature reports that CB1 receptors are mainly located in the central and peripheric nervous system, but it also has been found partially expressed in periodontal tissue as well as testis, retina, bone, adipocytes, heart, lung, prostate, spleen, uterus and ovaries.[18,33] It may be speculated that the CB1 receptors detected in the gingiva might have been expressed by peripheral nervous terminations or local immune cells.[34] On the other hand, the upregulation of CB2 receptors is not surprising, since they are expressed by cells originating from the hematopoietic/immune system, periodontal tissue and bone, playing a role in the mediation of inflammatory response.[21,26,35,36] The novelty of the present study is the assessment that CBs may be involved in the recurrence tendency of the disease since inflamed sites of recurrent patients had significantly higher CBs amount than other groups (HH and CD). This much higher CBs expression in inflamed recurrent PD sites may indicate a higher susceptibility of the tissue to activate the protective cellular/molecular mechanisms against periodontal destruction that follows bacterial infection. By contrast, in patients that present a recurrent disease despite their good oral hygiene level, the nature of this condition may indicate that these higher expressed receptors would be ineffective at protecting the tissue from inflammation.

Concerning the activation of CBs receptors, we observed a significantly lower percentage of active CBs receptors in inflamed sites of non-recurrent PD patients compared to healthy subjects. Otherwise, recurrent PD patients expressed an intermediate number of active receptors between that of healthy subjects and CD sites without significant differences with both. Since the active conformation of these receptors is related to their binding with the agonist, a greater CBs activation in presence of higher levels of AEA would be expected. On the contrary, data from the present study showed that the number of receptors activated in CD sites was lower than in HH, despite the higher levels of the agonist. Thus, it could be speculated that activity-related conformational changes or desensitization of receptors might occur in CD sites, leading to their inactive state. This data supports the importance to investigate the activation of CBs receptors together with the number of lipidic mediators when the endocannabinoid system is studied.

Concerning anandamide, it seems that even this molecule is implicated in the recurrent feature of the disease. In patients with recurrent periodontitis the inflamed sites (RD) had significantly lower levels of AEA than in non-recurrent patients (CD), but similar levels to subjects without periodontal disease (HH). It seems that patients that periodically show periodontal disease reactivation have a deficit in AEA production during inflammation. The literature reports the involvement of AEA during chronic periodontal inflammation and its role in the protection of periodontal

tissue against excessive inflammation by regulating cellular pathways leading to inflammatory responses as well as in the promotion of periodontal healing.[22,24,25,37] Nakajima et al showed that AEA concentration in periodontally diseased patients was 4 times higher than in healthy subjects and that this protein significantly reduced the release of pro-inflammatory cytokines (IL-6, IL-8 and MCP-1) produced by gingival fibroblasts after bacteria stimulation, through the activation of CB1 and CB2 receptors.[22] In a rodent model, Rettori et al (2012) assessed that the local injection of AEA reduced the levels of pro-inflammatory cytokines in experimental periodontitis even in stressed animals.[25] Kozono et al (2010) analyzed the granulation tissue obtained from human periodontal sites and biopsy blocks obtained from periodontal experimental wounds in rats. The authors observed that during periodontal healing the proliferation of gingival fibroblast may occur via CB1/CB2 receptors. It was also assessed that in granulation tissue from the periodontal healing sites, increased levels of AEA and upregulation of CBs receptors occurred, thus suggesting their important modulatory role in periodontal wound healing.[24]

Considering the protective role of the binding AEA/CBs receptors and since recurrent PD subjects of the present study expressed high levels of activated CBs but low levels of AEA, it would be hypothesized that this down-regulation may impair the innate response against the colonization and persistence of pathogens, thus making the subject more vulnerable to the reactivation of periodontal disease. To test this hypothesis, a second gingival biopsy was harvested after tissue healing and its AEA content was analyzed (RH, CH). It resulted that after non-surgical treatment AEA levels of recurrent PD subjects (RH) dropped significantly below the AEA levels of both healthy and non-recurrent PD patients. On the contrary, AEA levels of non-recurrent PD patients (CH), dropped to similar values to those observed in healthy subjects. These data seem to confirm that the capacity of cells to produce and release endocannabinoids protects the tissue from the reactivation of periodontal inflammation and that subjects with low basal levels of AEA may be predisposed to the disease reactivation. Furthermore, it would be speculated that during inflammation, the high expression of CB1/CB2 in the recurrent group may answer to a need of the tissue to compensate for the low AEA levels. It would be interesting to assess if the administration, during non-surgical treatment, of CB2 agonists, which showed potent anti-inflammatory action, to patients with up-regulation of these receptors (such as recurrent PD) may improve tissue healing and prevent the disease reactivation.[21,26] Data of the present study support the involvement of both the receptors and AEA in the pathogenetic mechanism of periodontal disease, and, to our knowledge, this is the first demonstration that a lack of AEA may prepare for the reactivation of the disease.

The endocannabinoid system is an innovative target for the development of anti-inflammatory and bone regenerative therapeutic strategies.[16] The use of CB2 agonists as anti-inflammatory agents for the regulation of periodontal chronic inflammation has been proposed in in vitro research.[21] Studies also proposed the reduction of

bone resorption during inflammatory disease or even the increase in bone regeneration by stimulating osteoblast formation or by inhibiting osteoclastogenesis through the activation of the receptor system. CB1 and CB2 stimulation and modulation were proposed in the literature to enhance bone formation and increase bone mineral density, however, conflicting data and the need for further studies emerged.[38–41] Data of the present study suggest a different predisposition between recurrent and non-recurrent patients to respond to the local application of CBs agonists, thus supporting a personalized therapeutic approach to PD. In patients with non-successfully treated disease, such as those belonging to the recurrent PD group of the present study, the local application of these ligands could be proposed, in particular of the CB2-selective agonists. In contrast, in patients with periodontal chronic inflammation, the application of anandamide ligands may not be resolutive. In fact, levels of AEA in CD patients were significantly higher than in HH; differently, the receptor activation was significantly lower than in HH, indicating the improvement of the CBs activation as a possible therapeutic strategy for CD patients rather than AEA administration. This consideration is furtherly supported by the evidence that AEA up-regulates pro-inflammatory cytokines in human periodontal fibroblasts stimulated with IL-1β, thus sustaining the disease progression.[26]

Among the limitations of the present study, there is the absence of data on the amount and activation of CB1 and CB2 receptors in the healed sites (T2) of recurrent and non-recurrent PD subjects. The decision to analyze only AEA in T2 biopsies was taken as a consequence of both the preliminary data on T1 samples that revealed the lack of this mediator in recurrent PD subjects and the necessity to limit the area of the harvested tissue for ethical reasons. Moreover, we did not investigate further molecules involved in the ES, such as the 2-Arachidonoylglycerol. This ligand was not assessed due to technical limitations concerning its analysis and the lack of literature on its role in periodontal disease. In fact, 2AG quantification at baseline is quite difficult by GC–MS/MS because its derivatization is by far less effective than AEA derivatization and needs elongated time, and shows a potentially increased risk for 2AG/1AG isomerization.[42,43]

5 | CONCLUSION

To conclude, data from the present human study show that:

1. In sites with recurrent and non-recurrent periodontitis the endocannabinoid system is differently involved;
2. In inflamed sites with recurrent disease: (i) the number of CBs was significantly higher than in non-recurrent and in healthy sites (ii) the levels of anandamide were comparable to those of healthy sites and lower than in inflamed non-recurrent sites, (iii) the number of active receptors was similar to that of healthy and non-recurrent sites;
3. In inflamed sites with non-recurrent disease: (i) the number of CBs was lower than in inflamed recurrent sites and higher than

in healthy sites, (ii) the levels of anandamide were higher than in healthy and inflamed recurrent sites, (iii) the number of active receptors is lower than in healthy sites;

4. After non-surgical treatment, anandamide levels of recurrent PD subjects dropped significantly below the AEA levels of both healthy and non-recurrent PD patients.

From a clinical perspective, a low level of anandamide may indicate a predisposition for the reactivation of the disease in patients that adhere rigorously to the periodontal maintenance program and comply with a satisfactory domestic oral hygienic program.

AUTHOR CONTRIBUTIONS

Gaia Pellegrini: Analysis; Drafting the work; interpretation of data for the work, Final approval of the version to be published; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Daniela Carmagnola: Design of the work; acquisition, revising the work critically for important intellectual content; Final approval of the version to be published; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Marilisa Toma: Acquisition, revising the work critically for important intellectual content; Final approval of the version to be published; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Giulio Rasperini: Acquisition, revising the work critically for important intellectual content; Final approval of the version to be published; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Marica Orioli: Acquisition, revising the work critically for important intellectual content; Final approval of the version to be published; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Claudia Dellavia: Conceptualization and design, revising the work critically for important intellectual content; Final approval of the version to be published; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST STATEMENT

The Authors declare no conflict of interest.

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