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The antimicrobial peptide LL-37 is anti-inflammatory and proapoptotic in human periodontal ligament cells

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Background and Objective: The antimicrobial peptide LL-37 is expressed in periodontal tissue, and variations in LL-37 levels have been associated with periodontal disease. The effects of LL-37 on periodontal ligament cell function have not been described before. Here, we assess anti-inflammatory properties of LL-37 and investigate the effects of LL-37 on cell differentiation, cell proliferation and apoptosis in human periodontal ligament cells.

Material and Methods: Periodontal ligament cells were obtained from teeth extracted for orthodontic reasons. Cytokine (interleukin-6) and chemokine (monocyte chemoattractant protein-1) expression was determined by quantitative PCR, cell differentiation by alkaline phosphatase activity, cell proliferation by counting cells in a Bürker chamber, DNA synthesis by incorporation of radiolabeled thymidine and apoptosis by cell morphology and activated caspase 3 quantities.

Results: Treatment with 0.1 and 1 μM of LL-37 totally reversed lipopolysaccharide-induced monocyte chemoattractant protein-1 expression and suppressed lipopolysaccharide-induced interleukin-6 expression by 50–70%. LL-37 had no effect on alkaline phosphatase activity. Incubation with 8 μM LL-37 strongly reduced cell number. DNA synthesis was attenuated by about 90% in response to 8 μM LL-37, confirming its antiproliferative effect. Cell morphology was altered in an apoptosis-like fashion in cells treated with 8 μM LL-37. Furthermore, the quantity of activated caspase 3 was increased in cells treated with 1 and 8 μM of LL-37, suggesting apoptosis.

Conclusion: LL-37 strongly attenuates lipopolysaccharide-induced cytokine and chemokine expression and, in high concentrations, reduces cell proliferation through inhibition of DNA synthesis and by promoting apoptosis in human periodontal ligament cells.

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The human cathelicidin LL-37 is a member of a large family of cationic antimicrobial peptides expressed in many species, in which it plays a central role in mucosal defense. Cathelicidin contains a signal peptide, a cathelin-like domain and an antimicrobial domain. The antimicrobial domain is released by cleavage by proteases; this domain is termed LL-37 (1). LL-37 exerts a direct antimicrobial activity by disrupting the wall of gram-negative and gram-positive bacteria (2,3), as well as by neutralizing lipopolysaccharides (LPS) (4). In the periodontium, neutrophils, monocytes and epithelial cells produce LL-37 (1).

Alterations in LL-37 levels have been associated with several pathological conditions. High levels of LL-37 have been reported in autoimmune diseases, such as psoriasis (5) and rosacea (6) and in ulcerative colitis (7), while low levels of LL-37 have been associated with infectious diseases, such as tuberculosis (8) and urinary tract infection (9), and with chronic ulcers (10). LL-37 is expressed in the periodontal tissue (11–13). Interestingly, there seems to be a lower expression of LL-37 in aggressive than in chronic periodontitis (12,13) and, furthermore, individuals lacking LL-37 due to morbus Kostmann disease are suggested to develop aggressive periodontitis consequently (14).

Besides its antibacterial effect, LL-37 has also been reported to affect other cellular functions, such as cell differentiation (15), phagocytosis (8) and apoptosis (16–21). Interestingly, the effects of LL-37 on apoptosis seem to be cell specific; in keratinocytes (17) and dermal fibroblasts (18) LL-37 suppresses apoptosis, but in vascular smooth muscle cells (16), airway epithelium (21), neutrophils (19) and T cells (20) LL-37 is reported to induce apoptosis.

Periodontal ligament cells are fibroblasts in the periodontal ligament responsible for the extracellular matrix turnover. Additionally, periodontal ligament cells will produce cytokines in response to LPS stimulation (22–24), thus it is reasonable to assume that they are important in the inflammatory response of the periodontium as well (25). As as result of the disease-associated alterations in LL-37 levels in the periodontium and the importance of periodontal ligament cells in both health and disease, it is essential to elucidate the cellular mechanisms regulated by LL-37 in periodontal ligament cells.

The hypothesis of this project is that LL-37 affects the periodontium by having an impact on the normal physiological properties of periodontal ligament cells as well as their inflammatory response. In order to test this hypothesis, we investigate the effects of LL-37 on LPS-induced cytokine and chemokine expression, cell differentiation, cell proliferation and apoptosis in human periodontal ligament cells.

Material and methods

Cell culture

The periodontal ligament was scraped off from the middle third of the root surfaces of premolars extracted for orthodontic reasons. The patients and their parents were informed, and a written consent was signed. Periodontal ligament tissue from five teeth originating from three individuals (two girls and one boy, aged 15, 16 and 24 years of age) were used. All experiments were performed in periodontal ligament cells originating from at least two clones. The study was approved by the Human Ethical Committee at Lund University. The tissue explants were seeded in culture dishes in Dulbecco's modified Eagle's medium supplemented with antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), glutamine (1.16 g/L) and 10% fetal calf serum (FCS). The cell culture dishes were placed in a water-jacketed cell/tissue incubator with 5% CO_2 in air, and the cells were allowed to migrate from the explants. The cells were trypsinized (0.25%) when reaching confluence and used for experiment in passages three to five. The immortalized MG63 osteoblast cell line (ATCC, Manassas, VA, USA) was cultured in the same conditions as periodontal ligament cells.

The concentrations of LL-37 (0.1, 1 and 8 μM) used in this paper are similar to those previously used in numerous papers (8,16,21,26,27), and these concentrations also correspond well to those observed in periodontal tissue (11).

Drugs

Escherichia coli LPS (Sigma, St Louis, MO, USA) was dissolved in phosphate-buffered saline. Nonamidated LL-37 (Bachem, Weil am Rhein, Germany) was dissolved in dimethyl sulfoxide. Control cells received the appropriate vehicle.

Quantitative PCR

Cells were grown for 24 h in the presence of vehicle or 1 μg/mL LPS, with or without 0.1 or 1 μM LL-37. Total RNA was extracted using the QIAshredder and RNeasy Mini kit (Qiagen, Valencia, CA, USA). The quantity and quality of RNA were measured on a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). The reverse transcription and quatitative PCR were done in one step using the QuantiFast SYBR green RT-PCR kits (Qiagen) on a Step One Plus Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The gene expression of monocyte chemoattractant protein-1 (*MCP-1*) and interleukin-6 (*IL-6*) in relation to the housekeeping gene 18S was calculated using the $\Delta\Delta Ct$ method. Each sample was analysed in triplicate. The PCR primers (QuantiTect Primer Assays) for *MCP-1*/chemokine ligand 2 (*CCL2*) (Hs_CCL2_1_SG), *IL-6* (Hs_IL6_1_SG) and 18S ribosomal RNA (Hs_RRN18S_1_SG) were from Qiagen.

Alkaline phosphatase activity assay

The cells were cultured in dextran-coated charcoal-stripped FCS and phenol red-free medium in order to rule out any osteogenic effects from steroids in the serum and estrogen-like effects of phenol red. The cells were stimulated for 7 d with LL-37; for the first 5 d the medium was supplemented with 10% FCS and for the final 2 d the cells were grown without serum. After 7 d of culture, the cells were scraped off the cell culture dishes using a rubber policeman. The cells were lysed by sonication over ice (twice for 10 s), spun down (1700*g*, 4°C for 5 min) and the supernatant was used for further analysis. Alkaline phosphatase (ALP) activity was measured by incubating the supernatant with 1 mg/mL 4-nitrophenyl phosphate (Sigma-Aldrich, St Louis, MO, USA) at 37°C for 2 h and was read on a colorimetric plate reader. Alkaline phosphatase activity was normalized to the amount of total protein in each sample determined by the Bradford assay (Bio-Rad, Hercules, CA, USA).

Cell proliferation and morphology

The cells were grown in medium without FCS for 24 h to make them

quiescent. Subsequently, this medium was replaced with a medium containing a submaximal growth-stimulating concentration (5%) of FCS. The cells were treated with LL-37 (0.1–8 μM) for 7 d. On day 7, the cells were washed and trypsinized, and the number of cells in each well was determined by counting the cells in a Bürker chamber. Cell morphology was assessed in digital photographs using a Nikon TMS microscope equipped with a digital camera (Pixelink, Nikon; Nikon Nordic AB, Solna, Sweden).

Measurement of DNA synthesis

Periodontal ligament cells were cultured in medium supplemented with a submaximal dose of FCS (5%), which causes a fourfold increase in DNA synthesis compared with cells cultured in serum-free medium at 24 h (28). In our previous work, we have observed that the impact of both 17β-estradiol (29) and LPS (23) on DNA synthesis is unaltered when the time of incubation is extended beyond 24 h, thus DNA synthesis was measured after 24 h of incubation with or without LL-37. During the final hour of incubation with or without LL-37 (0.1–8 μM), methyl-[^{3}H]thymidine (10 μCi; Perkin Elmer, Boston, MA, USA) was allowed to incorporate into newly synthesized DNA. The protocol has previously been described by Jönsson *et al.* (29). In short, the cells were washed in phosphate-buffered saline, scraped off using a rubber policeman, centrifuged and sonicated in 5 mM NaOH. After centrifugation, the supernatant was precipitated in 5% trichloroacetic acid and pelleted. The pellet was washed and dissolved in soluene. Liquid scintillation cocktail was added and the radioactivity measured in a liquid scintillation counter (Beckman LS6500; Beckman Instruments Inc., Fullerton, CA, USA).

Quantification of active caspase 3

After 24 h of treatment with or without LL-37 (0.1–8 μM), the cells were washed in phosphate-buffered saline, and protein was subsequently extracted from periodontal ligament cells using the protein extraction buffer provided in the Active Caspase 3 (Casp 3) ELISA kit (R&D Systems, Minneapolis, MN, USA). The ELISA was read colorimetrically on a plate reader and the quantity of active Casp 3 was normalized to the concentration of total protein in each sample determined using NanoDrop 2000.

Statistics

Values are presented as means ± SEM. Student's two-tailed *t*-test for unpaired data was used to determine statistical significance, with Bonferroni correction for multiple comparisons as appropriate. Values of $p < 0.05$ were regarded as statistically significant.

Results

Lipopolysaccharide-induced *MCP-1* and *IL-6* are suppressed by LL-37 in periodontal ligament cells

The effect of LL-37 on the LPS-mediated periodontal ligament cell inflammatory response was investigated by measuring *MCP-1* (*CCL2*) and *IL-6* gene expression in cells treated with 1 μg/mL *E. coli* LPS with or without 0.1 or 1 μM LL-37 for 24 h. Lipopolysaccharide increased *MCP-1* mRNA by about two times compared with untreated cells (Fig. 1A). Both 0.1 and 1 μM of LL-37 totally reversed LPS-induced *MCP-1* expression, as shown in Fig. 1A.

Lipopolysaccharides increased *IL-6* gene expression by about eight times compared with untreated control cells (Fig. 1B). LL-37 (0.1 μM) attenuated LPS-induced *IL-6* expression by about 50% (Fig. 1B). A higher concentration of LL-37 (1 μM) reduced LPS-induced *IL-6* by about 70%, as demonstrated in Fig. 1B.

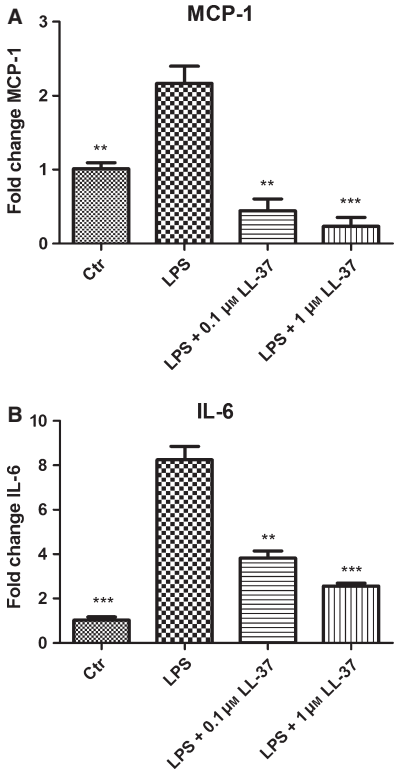

Fig. 1. Treatment with LL-37 (0.1 and 1 μM) totally reverses the lipopolysaccharide (LPS)-induced (1 μg/mL *E. coli* LPS) pro-inflammatory effect on monocyte chemoattractant protein-1 (*MCP-1*; A) and substantially attenuates the interleukin-6 (*IL-6*) gene expression (B) in periodontal ligament cells. The cells were treated with LPS with or without LL-37 for 24 h. The bars represent the mean and the error bars SEM of four observations in each group. $^{**}p < 0.0033$ and $^{***}p < 0.00033$ (Bonferroni corrected) compared with cells treated with LPS alone.

No effect of LL-37 on periodontal ligament cell osteogenic differentiation

The effect of LL-37 on osteogenic differentiation in periodontal ligament cells was assessed by measuring the ALP activity. Treatment with 0.1–8 μM LL-37 for 7 d had no effect on ALP activity (data not shown). The ALP activity was normalized to the total amount of protein in each sample. The effects of LL-37 (0.1–8 μM) treatment for 7 d on ALP activity were investigated in MG63 osteoblast cells as well. LL-37 had no effect on ALP activity in MG63 osteoblasts either (data not shown).

Treatment with LL-37 affects periodontal ligament cell morphology

Treatment with 8 μM LL-37 for 7 d affected periodontal ligament cell morphology and reduced cell density compared with control cells (Fig. 2).

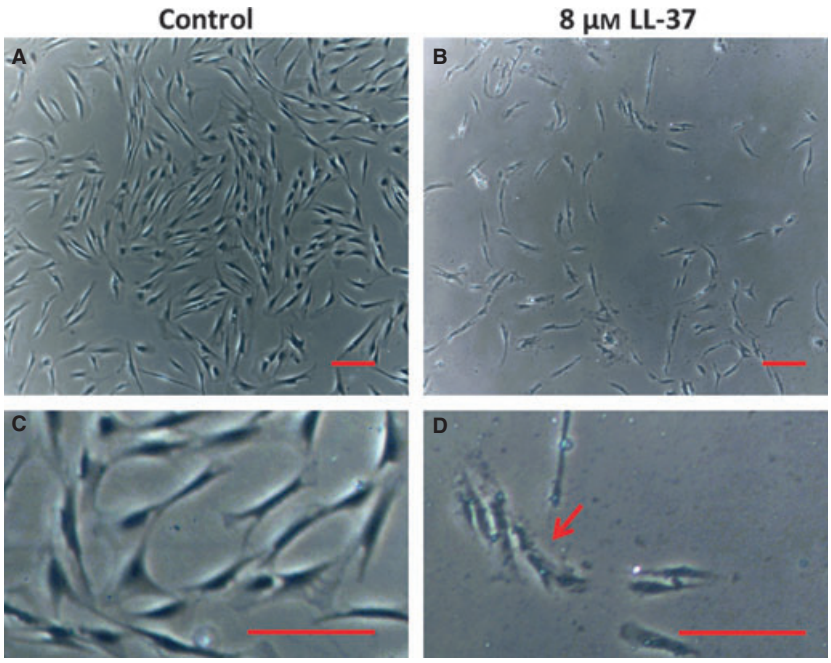

Fig. 2. Treatment with 8 μM LL-37 for 7 d reduces periodontal ligament cell density (A and B) and causes cell shrinkage and membrane blebbing (C and D), which are morphological characteristics of apoptosis. A representative cell is indicated by the arrow in D. Red bars represent 5 μM.

The LL-37-induced alteration of cell morphology was characterized by shrinkage of the cell and membrane blebbing as shown in Fig. 2. These morphological characteristics are classical signs of apoptosis (16). The lower concentrations (0.1 and 1 μM) of LL-37 had no effects on cell morphology and cell density (data not shown).

LL-37 suppresses periodontal ligament cell number and total protein content

Effects of treatment with LL-37 for 7 d on cell proliferation were assessed by measuring cell number and total cellular protein. LL-37 attenuated periodontal ligament cell number at 8 μM by about 90% (Fig. 3A). Lower concentrations of LL-37 (0.1 and 1 μM) tended to reduce cell number, but the effect was not significant (Fig. 3A).

Treatment with 8 μM LL-37 for 7 d reduced total cellular protein content by about 60% (Fig. 3B). The protein content was unaltered in cells treated with 0.1 and 1 μM LL-37 (Fig. 3B).

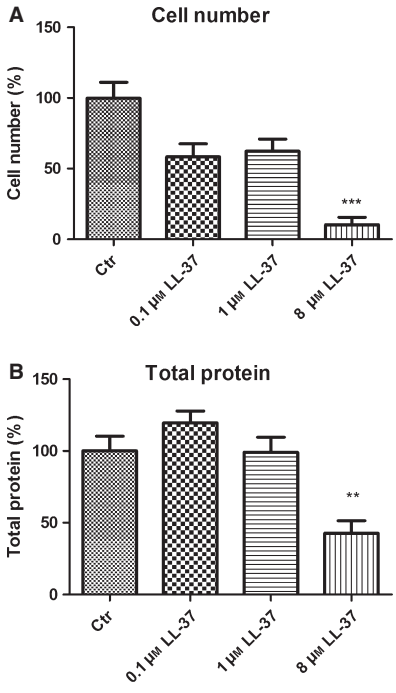

Fig. 3. Treatment with 8 μM LL-37 reduces cell number (A) and total amount of cellular protein (B) in periodontal ligament cells. The bars represent the mean and the error bars SEM of five to seven observations in each group. $**p < 0.0033$ and $***p < 0.00033$ (Bonferroni corrected) compared with control (Ctr).

LL-37 supresses DNA synthesis in periodontal ligament cells

To investigate the impact of LL-37 on DNA synthesis, periodontal ligament cells were stimulated with or without 0.1–8 μM of LL-37 for 24 h. Treatment with 8 μM LL-37 reduced DNA synthesis by about 90% compared with control cells, as shown in Fig. 4. Lower concentrations (0.1 and 1 μM) of LL-37 had no effects on DNA synthesis (Fig. 4).

LL-37 is proapoptotic in periodontal ligament cells

To investigate the impact of LL-37 on apoptosis an active Casp 3 ELISA was performed. periodontal ligament cells were stimulated with or without 0.1–8 μM LL-37 for 24 h. Both 1 and 8 μM LL-37 significantly increased the acti-

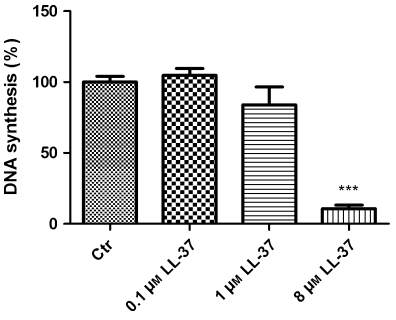

Fig. 4. Treatment with 8 μM LL-37 for 24 h reduces periodontal ligament cell DNA synthesis. The bars represent the mean and the error bars SEM of eight observations in each group. $***p < 0.00033$ (Bonferroni corrected) compared with control (Ctr).

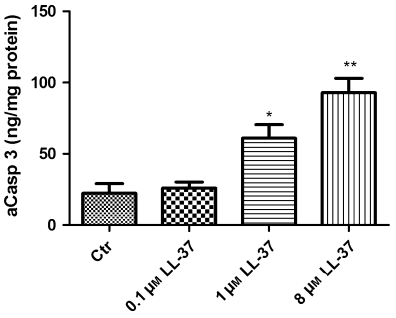

Fig. 5. Treatment with 1 and 8 μM LL-37 for 24 h increases the quantitiy of active caspase 3 (aCasp 3). The bars represent the mean and the error bars SEM of four observations in each group $*p < 0.0167$, $**p < 0.0033$ (Bonferroni corrected) compared with control (Ctr).

vation of Casp 3, as shown in Fig. 5. The lowest concentration of LL-37 (0.1 μM) had no effect on the activation of Casp 3.

Discussion

Here, we disclose that the antimicrobial peptide LL-37 reduces LPS-induced inflammatory responses of periodontal ligament cells, as well as attenuating periodontal ligament cell number by suppressing DNA synthesis and by inducing apoptosis. Interestingly, the anti-inflammatory effect of LL-37 starts at a low concentration (0.1 μM, corresponding to 0.45 μg/mL), while the proapoptotic effect of LL-37 starts at 1 μM and the antiproliferative effect at an even higher concentration (8 μM) of LL-37 (Fig. 6). When compared with the levels of LL-37 reported in the gingival crevicular fluid by Turkoglu *et al.* (11), 0.1 μM corresponds to the lower second quintile of healthy subjects (Fig. 6). One micromolar LL-37 corresponds to first higher quintile of patients with chronic periodontitis and the fouth higher quintile in healthy subjects. Eight micromolar LL-37 is high when compared with the levels in gingival crevicular fluid; however, the LL-37 level in the periodontal tissue is plausibly higher than 8 μM, in particular when the tissue has a high density of LL-37-rich neutrophils. Thus, both LL-37-induced anti-inflammation and antiproliferation observed in the present study are observed at physiologically and pathophysiologically relevant concentrations of LL-37.

Here, we report for the first time that LL-37 reduces proinflammatory properties of human periodontal ligament cells. LL-37 has been shown previously to reverse the inflammatory response induced by LPS, although not in periodontal ligament cells. In gingival fibroblasts, 0.1 μM LL-37 does not affect the LPS-induced (100 ng/mL) *IL-6* or interleukin-8 (IL-8) protein production; 1 μM LL-37, however, substantially reduces *IL-6* and IL-8 (30). It has also been reported that LL-37 (0.1 and 1 μM) attenuates heat-shock-killed *Porphyromonas gingivalis*-induced *IL-6* and IL-8 protein production in gingival fibroblasts (31). Here, we report that 0.1 and 1 μM LL-37 reverses the 1 μg/mL *E. coli* LPS-induced *MCP-1* and *IL-6* expression in human primary periodontal ligament cells, suggesting that LL-37 has similar effects on LPS-induced cytokine expression in gingival and periodontal fibroblasts. Several mechanisms may underpin the inhibitory effect of LL-37 on LPS signaling, as reported in the present study. These include the following mechanisms: (i) neutralization of LPS by LL-37 and interaction with the LPS-binding components of the Toll-like receptor 4 that reduces downstream activation (4,32); (ii) inhibition of LPS-induced translocation of nuclear factor-κB subunits p50 and p65 (26); and (iii) directly triggering MAPK pathways that may interfere with proinflammatory pathways (33).

Periodontal ligament cells are defined as fibroblasts with osteoblastic features. The ability of periodontal ligament cells to affect the metabolism of the alveolar bone and cementum has been considered important in the process of periodontal regeneration (34). Here, we report that LL-37 has no effect on periodontal ligament cell or osteoblast ALP activity, suggesting that LL-37 does not promote osteogenic differentiation in these cell types. LL-37 has, however, recently been reported to induce osteogenic differentiation in monocytes (15). Taken together, these data suggest that the capacity of LL-37 to stimulate osteogenic differentiation is cell type specific.

In the present study, we show that LL-37 reduces periodontal ligament cell number not only by suppressing DNA synthesis, but also by promoting apoptosis. In respect of gingival cells, LL-37 has been reported to reduce gingival keratinocyte and fibroblast cell number; however, the cause of the reduction in cell number was not further investigated (35). The proapoptotic effects of LL-37 in periodontal ligament cells are demonstrated both by morphological effects, such as cell shrinkage and membrane blebbing typical of apoptosis, and by enhanced quantities of activated Casp 3. LL-37 has been shown to be proapoptotic in vascular smooth muscle cells (16), neutrophils (19) and regulatory T cells (20) and to suppress apoptosis in keratinocytes (17) and in dermal fibroblasts (18), suggesting that the effect of LL-37 on apoptosis depends on cell type.

In conclusion, our data show that LL-37 substantially attenuates the periodontal ligament cell expression of *MCP-1* and *IL-6* induced by LPS and can therefore be considered anti-inflammatory in this experimental system. Importantly, LL-37 not only affects inflammatory properties of periodontal ligament cells, but also reduces cell proliferation by inhibiting DNA synthesis and stimulating apoptosis.

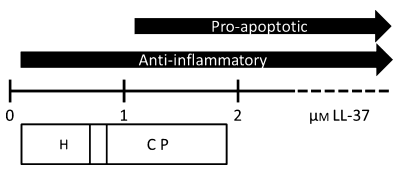

Fig. 6. The antimicrobial peptide LL-37 is anti-inflammatory in concentrations associated with periodontal health (H), but proapoptotic in concentrations associated with chronic periodontal disease (C P).The width of the boxes represents the upper and lower quartile of LL-37 in gingival crevicular fluid in health and chronic periodontitis (11).

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