Title: Antimicrobial and anti-inflammatory activity of Cystatin C on human gingival fibroblast incubated with *P. gingivalis*

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Abstract

Background. Periodontal disease is considered one of the most prevalent chronic infectious diseases, often leading to the disruption of tooth-supporting tissues, including alveolar bone, causing tooth mobility and loss. *Porphyromonas gingivalis* is considered the major etiological agent of this disease, having a plethora of virulence factors, including, lipopolysaccharides, hemolysins, and proteinases. Antimicrobial peptides are one of the main components of the innate immune response that inhibit the growth *of P. gingivalis***.** The aim of this study was to analyze the antimicrobial activity of Cystatin C and to assess the effect on the inflammatory and anti-inflammatory cytokines, the production of reactive oxygen species, and in the release of nitric oxide by human gingival fibroblasts incubated with *P. gingivalis* in the presence and absence of cystatin C.

Methods. *P. gingivalis* ATCC 33277 was exposed to cystatin C for 24h and co-cultured with human gingival fibroblasts (HGFs) ATCC CRL-2014. The effect of cystatin on growth of *P.*

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gingivalis and HGFs was evaluated. Pro-inflammatory (TNFα, IL-1β) and anti-inflammatory (IL-10) cytokines were determined by ELISA in the supernatants of HGFs incubated with *P. gingivalis* exposed to cystatin C. Additionally, nitrites and reactive oxygen species (ROS) production were evaluated.

Results. Cystatin C inhibited the growth of *P. gingivalis* without affecting HGFs. Incubation of HGFs with *P. gingivalis* led to a significant increase of TNF-α and IL-1β. In contrast, HGFs incubated with *P. gingivalis* exposed to cystatin C showed a decreased production of both cytokines, whereas IL-10 was enhanced. Incubation of HGFs with *P. gingivalis* led to an increased NO and ROS production, which was reduced in the presence of the peptide.

Conclusions. Cystatin C inhibits the growth *of Porphyromonas gingivalis* and decreases the inflammatory cytokines, ROS, and NO production during infection of HGFs with *P. gingivalis*. Knowledge on the antimicrobial and immunomodulatory properties of cystatin C could aid in the design of new therapeutic approaches to facilitate the elimination of this bacterium to improve the treatment of periodontal disease.

Introduction

Periodontitis is a chronic infectious disease, characterized by an exacerbated inflammatory response and progressive loss of tooth supporting tissues (Könönen et al., 2019) *Porphyromonas gingivalis* is a periodontal pathogen bacterium implicated as a major, etiological agent in periodontitis (van Winkelhoff et al., 2002). This bacterium has been recovered from periodontal pockets in a high percentage (75.8%) of patients with periodontitis (Rafiei et al., 2017).

The most abundant cell types in periodontal connective tissues are gingival fibroblasts (GF), where they participate in the repair of periodontal tissues during inflammatory periodontal diseases (Lee, et al., 2013). GF also promotes periodontal wound healing (Smith et al., 2019; Baek et al., 2013).

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Furthermore, LPS of *P. gingivalis* increases their superoxide concentrations after exposure to human gingival fibroblasts (HGFs) (Gölz et al., 2014). Thus, these cells can also participate in the progression of periodontitis, inducing the release of inflammatory such as: nitric oxide, cytokines, reactive oxygen species (ROS), and nitric oxide (How et al., 2016; Kirkwood et al., 2007; Gölz et al., 2014; Herath et al., 2016).

Cytokines are involved in the initiation and progression of periodontal disease (Ramadan et al., 2020). Even though secreted cytokines promote the elimination of bacteria, the overproduction of pro-inflammatory cytokines may participate directly in periodontal breakdowns, such as the breakdown of collagen periodontal attachment loss, and alveolar bone resorption (Gabay, Lamachia & Palmer, 2010). TNF-α and IL-1β are the major secreted pro-inflammatory cytokines, that are important markers of periodontitis progression and severity. and they are also the main inducers of effector molecules that cause the breakdown of periodontal tissues (Gomes et al., 2016). TNF-α and IL-1β are produced by several cell types including dendritic cells, macrophages, periodontal ligament cells, osteoblasts, and gingival fibroblasts and can act as multifunctional molecules (Cheng et al., 2020). IL-1β promotes production of metalloproteinases (MMPs), which are involved in the extracellular matrix degradation and, in turn, bone resorption and periodontal tissue destruction (Aleksandrowicz et al., 2021). TNF-α, participates in the bone resorption process, inducing receptor activators for nuclear factor -κB (RANK) expression in osteoclast precursors and RANKL expression in osteoblast (Pan, Wang & Chen, 2019). In addition, TNF-α and IL-1β also induce reactive oxygen species (ROS) generation in periodontal tissue (Wang et al., 2014), where oxidative stress has been shown to be involved in periodontitis (Tomofuji et al., 2006; Maruyama et al., 2011).These pro-inflammatory mediators are required for the immune defense against bacteria, yet their uncontrolled activity leads the accumulation of ROS (superoxide radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen) (Gölz et al., 2014). Even though these products stimulate proliferation and differentiation of cultured human periodontal ligament fibroblasts at low concentrations, their presence in higher concentrations can induce pathogen killing and cytotoxic effects on periodontal tissues and pathogen killing (Chapple & Matthews 2007). Zhu et al. (2020) demonstrated that after the stimulation of HGFs with LPS, ROS production in mitochondria (mtROS) were significantly enhanced these results indicate that oxidative stress can be induced during periodontitis (Liu et al., 2021). It is noteworthy that *P. gingivalis* is resistant to oxidative

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burst killing due to its antioxidant enzymes, such as thiol peroxidases and rubrerythrin. Furthermore, these bacteria accumulate a hemin layer on the cell surface that protects the bacteria from oxidative stress (Wang et al., 2014; Henry, McKenzie, Robles & Fletcher, 2012). On the other hand, IL-10, an anti-inflammatory cytokine that suppresses the inflammatory responses (Al-Rasheed et al., 2003), also protects from tissue destruction by inhibiting both matrix metalloproteinases (MMPs) and receptor activators for nuclear factor-kB (RANK) systems, leading to the differentiation and activation of osteoclasts (Garlet et al., 2006). Stimulation with bacteria or bacterial components like LPS induce the production of inflammatory cytokines, such as interleukin 1, -6, -8, and nitric oxide (NO), in human monocytes, endothelial cells, macrophages, and gingival fibroblasts (Gutierrez-Venegas et al., 2005; Staudte et al., 2010; Gölz et al, 2014). *P. gingivalis* triggers the production of NO by activating the expression of inducible nitric oxide synthases (NOS) (Sun, et al. 2010; Brennan, Thomas, & Langdon, 2003). It is noteworthy that it can resist NO stress and maintain nontoxic intracellular NO concentrations (Zumf, 2002). Thus, a high concentration of NO fails to eliminate this bacterium, yet it can exert a deleterious effect on the periodontal tissue, favoring vasodilation and diminishing platelet aggregation, which contributes to gingival bleeding. These toxic effects on the surrounding tissue increase the severity of periodontitis (Boutrin et al., 2012). It has been suggested that the inducible nitric oxide synthase (iNOS) may be involved in periodontal disease (Batista et al., 2002), because usually periodontal pathogenic bacteria increase the production of iNOS, including HGFs (Sosroseno, et al., 2009). Furthermore, cytokines and chemokines produced by gingival fibroblasts in response to *P. gingivalis* infection could increase and their effects on leukocytes are modulated by the enzymatic activity of *P. gingivalis*-derived proteinases, that cleave and disrupt their functions (Calkins et al. 1998; Kobayashi, Isogi & Hirose 2003; Palm, Khalaf & Bengtsson, 2015). The production of *P. gingivalis* cysteine proteinases are associated with the growth and establishment of *P. gingivalis,* they are divided into arginine-specific (Rgp) and lysine-specific (Kgp) proteinases. Additionally, these cysteine proteases exert potent immunomodulatory effects on human gingival fibroblasts. The main causative factor of tissue damage involved in the disease progression, could be the gingipains of the bacterium, even though *P. gingivalis* is considered an opportunistic pathogen. Thus, control of proteolytic enzymes of *P. gingivalis* could represent an interesting target for the treatment of periodontitis (Torbjörn, Atika &Hazem, 2015).

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Antimicrobial peptides (AMPs) are part of the innate defense system in the oral cavity, where cystatins play an important role. Cystatin C belongs to the type 2 family of the cystatin superfamily, it is ubiquitously distributed in plants and animals (Shamsi & Bano, 2017). In the parotid gland of humans, it is present in saliva at a concentration of 0.9 µg/mL (Gorr, 2012). The main function of cystatin C is the inhibition of cysteine proteases by binding to their active sites (Palm, Khalaf & Bengtsson, 2015). It also exerts several immunomodulatory functions and possesses the ability to regulate innate immune responses (Vray, Hartmann & Hoebeke, 2002). The aim of this study was to assess the effect that cystatin C exerts on cytokine production, NO and ROS production by human gingival fibroblasts incubated with *P. gingivalis* in order to be able to evaluate its potential therapeutic use against one of the main etiological agents causing periodontitis, as well as its potential impact on the severity of periodontal disease.

Materials & Methods

Cells culture

Human gingival fibroblasts (HGFs) (ATCC, CRL-2104) were seeded at a density of 5×10^3 cells/cm^2 and cultured in 75 cm^2 culture flasks in water-saturated atmosphere at 37°C plus 5% CO_2 and maintained in Dulbecco´s modified Eagle high glucose medium (Sigma Aldrich, Saint Louis, MO, USA), supplemented with 10% fetal bovine serum (GIBCO BRL, Gaithersburg, MD, USA), containing 10 U penicillin plus 25 µg streptomycin /mL) (Sigma Aldrich). The fibroblasts were cultured to confluence, at a density of 2.5×10^5 cells/mL, washed twice with phosphate-buffered saline, and dissociated with 0.25% trypsin and 1 mM EDTA for 5 min at 37°C, 5% CO_2 (Sigma Aldrich, Saint Louis, MO, USA). The cells were used at passages 3-7.

Bacterial growth

P. gingivalis strain ATCC 33277 was cultured in brain-heart-infusion and in broth-heart-brain extract (BHI; BD Bioxon, Milan, Italy) containing 5 µg/mL of hemin (Sigma-Aldrich, Munich, Germany) supplemented with 1 µg/mL of menadione (Sigma-Aldrich) cultured at 37°C for 24 h in anaerobic conditions, using the BBL-GasPak jar system (BD Biosciences).

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After cultivation for 24 h, bacteria were recovered by centrifugation (10 min at 10000 rpm), washed and resuspended in Krebs-Ringer-Glucose (KRG) buffer (120 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, 10 mM glucose, and 1.1 mM $CaCl_2$, pH 7.3). Bacterial growth was monitored spectrophotometrically (Jenway Genova R0027, Fischer Scientific, USA) at 675 nm. The bacterial density was visually adjusted to a turbidity of 0.5 McFarland (1×10^8 colony-forming units (CFU/mL) (Mc Farland, 1907 Emani et al., 2014)). Ethical approval was given by the Ethics Committee of the School of Medicine (UNAM) with reference number C54-11.

Antibacterial assay

Lyophilized cystatin C was obtained from *Pichia Pastoris* (Sigma Aldrich, St. Louis, MO) and reconstituted in Tris Base NaCl Buffer (pH 7.4). Minimum inhibitory concentrations (MIC) of Cystatin C were determined using the microdilution method in 96-well microtiter plates (Costar, Corning Life Sciences) (Eloff, 1998; Jadaun et al., 2007). Briefly, an inoculum of *P. gingivalis* ($1x10^6$ CFU/mL) containing KRG Buffer was placed in each well. Subsequently, different cystatin C concentrations (0.1, 0.3, 0.5, 0.7, 0.9 µg/mL) were incubated with the bacteria for 1, 12, 24, and 48 h, under anaerobiosis conditions at 37°C. After the incubation period, 20 µL of Presto Blue Cell Viability Reagent (Invitrogen, Thermo Fisher Scientific) per well were added. The plates were incubated for 30 min at 37°C in the dark. Finally, the plates were read in a microplate reader (Multiskan SkyHigh Microplate Spectrophotometer) at a 675 nm wavelength.

Cell Viability assay

HGFs were seeded at a density of $1x10^5$ cells/well in 24-well plates for 24 h, at 37°C with 5% CO_2. Different concentrations of cystatin C (0.1, 0.3, 0.5,0.7,0.9 µg/ml) were added and incubated for 24 h. After incubation time, 25 µl of XTT/PBS solution (4 mg/4ml) were added per well, for 40 minutes at room temperature, in the dark. Subsequently, microplate plates were read at a wavelength of 450 nm in a microplate spectrophotometer (Multiskan SkyHigh Microplate Spectrophotometer).

Treatment of human gingival fibroblasts (HGFs) with *P. gingivalis*

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Human gingival fibroblasts, at a seeding density of 5×10^5 cells/well, were cultured in a Costar® 24-well plate (Corning Life Sciences, Corning, NY, USA) in DMEM medium at 37°C in an atmosphere of 5% CO_2. After the incubation period, fresh medium without antibiotics was added to HGFs, before they were treated with *P. gingivalis.* HGFs were stimulated with bacteria, at multiplicities of infection (MOI) of 1:100 for 24 h, and with cystatin C at a concentration of 0.3 µg/mL at 37°C for 24 h, to perform cytokine assays, and evaluate ROS, and NO. Control groups include HGFs without stimulation or stimulated with 100 ng/mL of LPS (LPS from *Escherichia coli* O111:B4, Sigma Aldrich), or with10µg/mL of peptidoglycans (Peptidoglycan from *Staphylococcus aureus*, Sigma Aldrich).

Cytokine assays

For cytokine assays, HGFs were incubated with *P. gingivalis* (MOI 1:100) and/or with cystatin C at a concentration of 0.3 µg/mL at 37°C for 24 h. Control groups included HGFs without stimulation or stimulated with LPS 100 ng/mL (LPS from *Escherichia coli* O111:B4, Sigma Aldrich) or with peptidoglycan 10 µg/mL (Peptidoglycan from *Staphylococcus aureus*, Sigma Aldrich). ELISAs were performed to determine TNF-α, IL-1β, and IL-10, using the Ready-Set-Go! ELISA kits (BD Biosciences, Cytokine ELISA Protocol, San Diego, CA, USA), following the manufacturer's protocol. Dilutions were prepared in dilution buffer. Briefly, 96-well plates with flat-bottom (Costar®, Corning Life Sciences) were coated with anti-human monoclonal antibodies recognizing IL-1β, IL-10 or TNF-α (BD Biosciences, Pharmingen). After blocking with the assay solution (PBS-0.5% casein diluted in 1 M NaOH) an overnight incubation at 4°C was done in order to eliminate non-specific binding, for this 100 µL of standard TNF-α, IL-1β, or IL-10 (BD Bioscience, Pharmingen) of supernatants were added. The microplate was washed to remove unbound enzyme-labeled antibodies. The amount of horseradish peroxidase in each well was revealed by the addition of a substrate solution. Finally, the reaction was stopped by the addition of 0.18 M sulfuric acid and the plates were read at 405 nm (ELISA microplate reader, Bio-Rad, Hercules, CA, USA).

The cytokine concentrations were calculated by regression analysis from a standard curve. The detection limit of the assay was 15 to 2000 pg/mL.

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Measurement of NO production

The NO production by HGFs incubated with *P. gingivalis* and/or cystatin C at 37°C was assayed by measuring the accumulation of nitrate in culture supernatants. Briefly, HGFs were stimulated with *P. gingivalis* (MOI 1:100) and with 0.3 µg of cystatin C, at 37°C for 24 h. Thereafter, 100 µL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 2.5% phosphoric acid) (Sigma Aldrich) were added at equal volumes of culture supernatants in a 96- well plate (Costar®, Corning Life Sciences) and left at room temperature for 30 min. The absorbance of these supernatants were read at 550 nm (Multiskan SkyHigh Microplate Spectrophotometer) and the nitrate concentrations were calculated from a standard curve established with serial dilutions of $NaNO_2$ (Sigma-Aldrich) in the culture medium. Control groups included HGFs without stimulation or stimulated with LPS or peptidoglycan.

Detection of Reactive Oxygen Species (ROS)

HGFs were seeded on 24-well plates (Costar®, Corning Life Sciences) at a density of ($5x10^5$), infected with *P. gingivalis* (MOI 1:100) and stimulated with 0.3 µg/ml of cystatin C at 37°C for 24 h. The cells were incubated with 100 µg/mL [2 µM/mL] of 2,7 dichlorodidrofluoroescein diacetate (H2-DCFDA) for 30 min in the dark at room temperature. Cells were rinsed twice with PBS, pH 7.2 and detached from the wells with 0.25% Trypsin/EDTA (Sigma Aldrich). The samples were resuspended in PBS, pH 7.2, with 1% FBS and analyzed on a FACS Canto II BD Biosciences flow cytometer. Data analysis was performed using FlowJo software (USA). Control groups included HGFs without stimulation or stimulated with LPS or peptidoglycan.

Statistical analysis

Experimental and control conditions were statistically compared for significance using analysis of variance (ANOVA), followed by Benferroni correction. The predetermined level of significance was $p < 0.05$. Statistical analysis was performed with the GraphPad, Prism v.6 software (GraphPad Software, Inc., CA, USA).

Results

Effects of cystatin C on growth of *P. gingivalis* and viability of HGFs

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The antimicrobial activity of cystatin C on *P.gingivalis* was analyzed in a time and dose-dependent manner as shown in (Fig. 1a). It reached its maximal antimicrobial activity at 24 h with concentrations between 0.1 and 0.3 µg/mL.

The concentration of 0.3 µg/mL inhibited 75% of bacteria growth after 24h of incubation when compared to the control group ($p < 0.05$). Inhibition of bacterial growth (83.3%) was observed after 48 h of culture ($p < 0.05$). At a concentration of 0.9 µg/mL a marked growth inhibition was observed throughout the incubation time. All the analyzed concentrations of cystatin C showed no effect on the viability of HGFs cells, as illustrated in (Fig. 1b). These findings reveal the antimicrobial activity of cystatin C against *P. gingivalis* and did not affect the viability of HGFs. Hence, we decided to perform all the experimental assays with a cystatin C MIC at 0.3 µg/mL.

Effect of cystatin C on the production of pro- and anti-inflammatory cytokines

TNF-α and IL-1β were evaluated in supernatants of HGFs incubated with *P. gingivalis* and cystatin C (0.3 µg/mL) for 24 h. *P. gingivalis* induced the production of 1000 pg/mL and 750 pg/mL of TNF-α and IL-1β, respectively, when compared to the control group ($p = 0.0001$) (Figs 2a, 2b). However, when HGFs were incubated with the bacteria and cystatin C, a statistically significant decrease was observed in the TNF-α ($p = 0.0001$) and IL-1β ($p < 0.05$) productions, compared to HGFs. In contrast, no changes were observed in IL-10 production by HGFs incubated with *P. gingivalis* alone, when compared to controls, whereas cystatin C stimulated de production and secretion of IL-10 (500 pg/mL). Furthermore, the co-incubation of *P. gingivalis* with cystatin C significantly increased the production of IL-10 (900 pg/mL), when compared with the control group and with HGFs infected with the bacterium ($p = 0.0001$), (Fig.2c). These results suggest that cystatin C participates in the regulatory inflammatory process, by reducing inflammatory cytokines and increasing anti-inflammatory cytokines.

Cystatin C decreases ROS and NO production on HGFs incubated with *P. gingivalis*

A significant increase was observed in the production of ROS and NO in HGFs incubated with *P. gingivalis*, compared to the controls ($p = 0.0001$). No significant differences were observed in the production of ROS in HGFs incubated with cystatin C ($p > 0.05$) (Fig.3a). In contrast, a significant decrease in ROS was observed after the incubation of HGFs with *P. gingivalis* and cystatin C, compared to the control ($p = 0.001$), (Fig. 3a).

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Furthermore, a significant increase of NO (9 µM) was observed after the incubation of HGFs with *P. gingivalis,* when compared with the control group (p = 0.0001). Yet when HGFs were incubated with *P. gingivalis* and cystatin C, a decrease of NO (3 µM) (p = 0.001) was observed regarding the incubation with *P. gingivalis* alone (Fig. 3b).

Discussion

In this study, we analyzed the antimicrobial activity of cystatin C against *P gingivalis*, which contributes to the development of chronic periodontitis. The immunological responses occurring in HGFs after the infection with this key periodontal pathogen were evaluated. *P. gingivalis* exhibits a variety of virulence factors that enable it to colonize oral soft tissues and evade immune responses. It has been demonstrated that *P. gingivalis* triggers and suppresses the immune responses in HGFs, suggesting that the pathogenic effects of *P. gingivalis* are mainly related to the action of gingipains, which participate in the inflammatory and immune response of HGFs (Palm, Khalaf & Bengtsson, 2015; Bengtsson, Khalaf & Palm, 2015). Additionally, *P. gingivalis* has a direct modulatory function on the immune response of fibroblasts through the catalytic activities of gingipains, targeting fibroblast-derived inflammatory mediators at the protein level (Palm, Khalaf & Bengtsson, 2013). *P. gingivalis* secretes three related cysteine proteases (gingipains), which constitute its main virulence factors. Two gingipains are specific for Arg-Xaa peptide bonds (HRgpA and RgpB), whereas Kgp cleaves after a Lys residue (Imamura, 2003). Interestingly, gingipains are involved in the disruption of host defense inflammatory reactions and hinder *P. gingivalis* clearance by the immune system (Uehara et al., 2008; Guo, Nguyen & Potempa, 2010). Human gingival fibroblasts play an important part in the innate immune system by sensing microbial invasion and responding to it by producing and secreting inflammatory mediators. HGFs recognize *P. gingivalis* during the early stages of periodontitis and establish an inflammatory response in the periodontal tissue (Palm, Half & Bengtsson, 2015). The secretion of TNF-α and IL-1β by HGFs favor the recruitment of macrophages and neutrophils to the site of infection, as well as the expression of MMP-1, MMP-13, MMP-8, and MMP-9, which contribute to the degradation of the extracellular matrix of the periodontal tissue as well as the reabsorption of bone tissue (Ara et al., 2009; Song et al., 2021; Cheng et al., 2020; Franco et al., 2017; Siu et al; 2020; Menaka et al., 2009).

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Interleukin-1β (IL-1β), belongs to the IL-1 family and plays an important role against microbial infections and participates regulating innate immune and inflammatory responses. The upregulation of IL-1β during *P. gingivalis* infection suggests that IL-1β is a critical cytokine in the host's defense against *P. gingivalis* infection during the initial phases of inflammation (Dinarello, 2009). In the early stages of *P. gingivalis* infection, IL-1β plays an important role in combating the invading pathogen as part of the innate immune response and participates in almost all events involved in the activation and regulation of inflammation (Menu & Vince, 2011). This kind of inflammasome-independent IL-1β activation can substantially contribute to tissue inflammation (Latz & Xiao & Stutz, 2013).

We now demonstrate that cystatin C down-regulates the production of IL-1β and TNF-α in HFGs co-incubated with *P. gingivalis*. Our finding is in accordance with the literature, where cystatin C has been shown to down-regulate the production of IL-1β and TNF-α in monocytes stimulated with bacterial LPS (Gren et al., 2016). In addition to cystatin C, other salivary antimicrobial peptides, such as histatin 5 and histatin 1, also down-regulate inflammatory cytokines like IL-6, IL-8, IL-1β, and TNF-α in fibroblasts and macrophages (Imatani et al., 2000; Lee et al., 2021). Our data also show that cystatin C enhances IL-10 production by HFGs incubated with *P. gingivalis*, which could represent an important mechanism to inhibit an excessive inflammatory response of HGFs to the *P. gingivalis* infections. The cytokine IL-10 can inhibit pro-inflammatory responses, due to its ability to reduce the production of TNF-α, IL-6, and IL-1 cytokines (Sun et al., 2020). Our results suggest that cystatin C could be an important multifunctional modulator of the innate immune responses in HGFs.

In addition to cytokine production, HGFs also produce microbicidal mediators such as ROS and NO, when they are infected with *P. gingivalis*. High doses of these molecules have been shown to be cytotoxic to periodontal tissue (Nogueira et al., 2016), since their excessive production may lead to tissue breakdown, including inhibition of energy-generating enzymes, triggering DNA injury, oxidation and nitration reactions, (Wang, Huang & He, 2019; Bodis & Haregewoin,1993). ROS causes oxidative damage to proteins and DNA, it interferes with cell growth, and induces apoptosis in gingival fibroblasts, causing periodontitis (Kanzaki et al., 2017; Cheng et al., 2015; Tomofuji et al., 2006; Marayuma et al., 2011). In addition to the damage caused by ROS, an increase of iNOS expression and NO concentration also leads to severe damage related to bone resorption, as shown in an experimental rat model of periodontitis

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(Wang, Huang & He 2019). Thus, many inflammatory mediators are crucial for the development of early periodontal disease, where NO is one of the main inflammatory factors (Pacher Beckman, & Liaudet, 2007). *P. gingivalis* induces NO production and inducible nitric oxide synthase (iNOS) expression in immune and nonimmune host cells (Sun et al., 2010). Although macrophages are the source of the iNOS expression, NO production is elevated in HGFs that are stimulated by TNF- α, IL-1β, and IFN-γ. NO high concentrations they have a side effect on the periodontal tissue, favoring vasodilation and platelet aggregation diminish, which can contribute to gingival bleeding, aside from having cytotoxic effects on the surrounding tissue, increasing the severity of the periodontitis (Boutrin et al., 2012).

Our data now demonstrated that *P. gingivalis* stimulates NO release by HGFs and that the co-incubation of the bacterium with cystatin C significantly down-regulates both ROS and NO productions. These findings are in accordance with the literature, showing that other peptides, such as hBD3 and sublancin, also reduce the production of ROS in endothelial cells and NO in peritoneal macrophages, respectively (Wang, Huang & He, 2019; Bian et al., 2017). The results of our study suggest that NO expression could lead to the gradual progression of periodontitis after proinflammatory cytokine production by HGFs infected by *P. gingivalis* and that cystatin C protects from tissue damage through the reduction of these free radicals. The importance of ROS in periodontal diseases was previously demonstrated by Cheng et al, who showed that LPS from *P. gingivalis* up-regulated ROS in periodontal ligament fibroblasts (Cheng et al., 2015; Goltz et al., 2014). The release of inflammatory mediators including interleukins, chemokines, adhesion molecules, and ROS could be.could be triggered by bacteria LPS (Goraca et al., 2013; Melo et al., 2010; Sanikidze et al., 2006; Bykov et al., 2003).

Antimicrobial peptides are included in the immune innate defense system in the oral cavity (Greer, Zenobia & Darveau 2013). The antimicrobial peptide cystatin C belongs to the type 2 family of the cystatin superfamily, it is ubiquitously distributed in plants, animals, and microorganisms (Shamsi & Bano, 2017). Saliva from the parotid gland of humans contain 0.9 µg/mL of cystatin C (Gorr S, 2009). The main function of cystatin C is the inhibition of cysteine proteases, by binding to their active sites, evading the cleavage of peptide bonds (van Wyk, et al., 2016). The mechanisms leading to the reduction of the inflammatory mediators by cystatin C are possibly explained by observations made with a homologous molecule, DsCistatin, isolated from the tick *Dermacentor silvarum*. This peptide was shown to be internalized by endocytosis

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in mouse macrophages stimulated with LPS from *Borrelia burgdorferi*. It reduced the inflammatory cytokines IL-1β, IFN-γ, TNF-α, and IL-6 by the degradation of the TRAF6 protein, thereby preventing the phosphorylation of IκBα and the subsequent nuclear transport of NF-κB, leading to the decrease of inflammatory cytokines (Sun et al., 2018). We speculate that cystatin C possibly follows this route to reduce inflammatory mediators in HGFs incubated with *P. gingivalis.*

Our data now show that cystatin C possibly plays an important antimicrobial and anti-inflammatory role that regulates the response of human gingival fibroblast towards *P. gingivalis,* helping to avoid tissue damage and destruction.

Conclusions

Cystatin C exhibits a dual activity during *P. gingivalis* infection. Antimicrobial activity was demonstrated without cytotoxic effects on HGFs. Furthermore, cystatin C also exhibited immunomodulatory functions, decreasing the inflammatory response of fibroblasts. Knowledge on the immunomodulatory properties of cystatin C could aid in the design of new therapeutic approaches to improve the treatment of periodontal diseases.

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Acknowledgements

We thank Drs Daniela Cortés Hernández and Dulce Verónica Rivero Gamallo for their assistance in the culture of bacteria and human fibroblasts during the initial phase of the study and Rocely Cervantes Sarabia for her assistance in cytotoxicity assays.

References

Aleksandrowicz P, Brzezińska-Błaszczyk E, Kozłowska E, Żelechowska P, Borgonovo A E, & Agier J. 2021. Analysis of IL-1β, CXCL8, and TNF-α levels in the crevicular fluid of patients with periodontitis or healthy implants. *BMC oral health*, *21*(1), 120. https://doi.org/10.1186/s12903-021-01478-3

Al-Rasheeda A, Scheerens H, Srivastava A K, Rennick D M, & Tatakis D N. 2004. Accelerated alveolar bone loss in mice lacking interleukin-10: late onset. *Journal of periodontal research*, *39*(3), 194–198. https://doi.org/10.1111/j.1600-0765.2004.00724.x

Ara T, Kurata K, Hirai K, Uchihashi T, Uematsu T, Imamura Y, Furusawa K, Kurihara S, & Wang P L. 2009. Human gingival fibroblasts are critical in sustaining inflammation in

periodontal disease. *Journal of periodontal research*, *44*(1), 21–27. https://doi.org/10.1111/j.1600-0765.2007.01041.x

Atanasova K, Lee J, Roberts J, Lee K, Ojcius DM & Yilmaz Ö. 2016. Nucleoside-Diphosphate-Kinase of P. gingivalis is Secreted from Epithelial Cells In the Absence of a Leader Sequence Through a Pannexin-1 Interactome. *Scientific reports*, *6*, 37643. https://doi.org/10.1038/srep37643

Baek KJ, Choi Y, & Ji S. (2013). Gingival fibroblasts from periodontitis patients exhibit inflammatory characteristics in vitro. *Archives of oral biology*, *58*(10), 1282–1292. https://doi.org/10.1016/j.archoralbio.2013.07.007

Bascones A, Gamonal J, Gomez M, Silva A, & Gonzalez M A. 2004. New knowledge of the pathogenesis of periodontal disease. *Quintessence international (Berlin, Germany : 1985)*, *35*(9), 706–716.

Bengtsson T, Khalaf A, & Khalaf H. 2015. Secreted gingipains from *Porphyromonas gingivalis* colonies exert potent immunomodulatory effects on human gingival fibroblasts. *Microbiological research*, *178*, 18–26. https://doi.org/10.1016/j.micres.2015.05.008

Bian T, Li H, Zhou Q, Ni C, Zhang Y, & Yan F. 2017. Human *β*-Defensin 3 Reduces TNF-*α*-Induced Inflammation and Monocyte Adhesion in Human Umbilical Vein Endothelial Cells. *Mediators of inflammation*, *2017*, 8529542. https://doi.org/10.1155/2017/8529542

Blancas B, Lanzagorta ML, Jiménez-Garcia LF, Lara R, Molinari JL, Fernández AM. 2021. Study of the ultrastructure of *Enterococcus faecalis* and *Streptococcus mutans* incubated with salivary antimicrobial peptides. Clin Exp Dent Res. 7(3):365-375. doi: 10.1002/cre2.430.

Blankenvoorde MF, van't Hof W, Walgreen-Weterings E, van Steenbergen TJ, Brand HS, Veerman EC & Nieuw Amerongen AV. 1998. Cystatin and cystatin-derived peptides have antibacterial activity against the pathogen Porphyromonas gingivalis. *Biological chemistry*, *379*(11), 1371–1375

Bodis S, & Haregewoin A. 1993. Evidence for the release and possible neural regulation of nitric oxide in human saliva. *Biochemical and biophysical research communications*, *194*(1), 347–350. https://doi.org/10.1006/bbrc.1993.1826

Boutrin MC, Wang C, Aruni W, Li X, & Fletcher H M. 2012. Nitric oxide stress resistance in *Porphyromonas gingivalis* is mediated by a putative hydroxylamine reductase. *Journal of bacteriology*, *194*(6), 1582–1592. https://doi.org/10.1128/JB.06457-

Brennan PA, Thomas GJ, Langdon JD. (2003).The role of nitric oxide in oral diseases. Arch. Oral Biol. 48(2) :93–10. https://doi.org/10.1016/S0003-9969(02)00183-8

Bykov I, Ylipaasto P, Eerola L, & Lindros K O. 2003. Phagocytosis and LPS-stimulated production of cytokines and prostaglandin E2 is different in Kupffer cells isolated from the periportal or perivenous liver region. *Scandinavian journal of gastroenterology*, *38*(12), 1256–1261. https://doi.org/10.1080/00365520310007116

Dio formato

Dio formato

CalkinsC C, Platt K, Potempa J, & Travis J. 1998. Inactivation of tumor necrosis factor-alpha by proteinases (gingipains) from the periodontal pathogen, *Porphyromonas gingivalis*. Implications of immune evasion. *The Journal of biological chemistry*, *273*(12), 6611–6614. https://doi.org/10.1074/jbc.273.12.6611

Chapple I L, Matthews J B. 2007. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. Periodontol. 2000 43, 160–232. 10.1111/j.1600-0757.2006.00178.x

Cheng R, Choudhury D, Liu C, Billet S, Hu T, & Bhowmick N A. 2015. Gingival fibroblasts resist apoptosis in response to oxidative stress in a model of periodontal diseases. *Cell death discovery*, *1*, 15046. https://doi.org/10.1038/cddiscovery.2015.46

Cheng R, Wu Z, Li M, Shao M, & Hu T. 2020. Interleukin-1β is a potential therapeutic target for periodontitis: a narrative review. *International journal of oral science*, *12*(1), 2. https://doi.org/10.1038/s41368-019-0068-8

Christopher J R, Ponnaiyan D, Parthasarathy H, & Tadepalli A. 2021. Association of *CST3* Gene with Its Protein: Cystatin C in Health and Severe Periodontal Disease. *Genetic testing and molecular biomarkers*, *25*(6), 405–410. https://doi.org/10.1089/gtmb.2021.0049

Dinarello C A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annual review of immunology*, *27*, 519–550. https://doi.org/10.1146/annurev.immunol.021908.132612

Eloff J N. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta medica*, *64*(8), 711–713. https://doi.org/10.1055/s-2006-957563

Emani S, Gunjiganur G V, &amp Mehta D S. 2014. Determination of the antibacterial activity of simvastatin against periodontal pathogens, Porphyromonas gingivalis and Aggregatibacter

actinomycetemcomitans: An in vitro study. Contemporary clinical dentistry, 5(3), 377–382. https://doi.org/10.4103/0976-237X.137959

Franco C, Patricia H R, Timo S, Claudia B, & Marcela H. (2017). Matrix Metalloproteinases as Regulators of Periodontal Inflammation. *International journal of molecular sciences*, *18*(2), 440. https://doi.org/10.3390/ijms18020440

Gabay C, Lamacchia C, & Palmer G. 2010. IL-1 pathways in inflammation and human diseases. *Nature reviews. Rheumatology*, *6*(4), 232–241. https://doi.org/10.1038/nrrheum.2010.4

Garlet G P, Cardoso C R, Silva T A, Ferreira B R, Avila-Campos M J, Cunha F Q, & Silva J S. 2006. Cytokine pattern determines the progression of experimental periodontal disease

Dio formato: Inglés (americano)

634 induced by *Actinobacillus actinomycetemcomitans* through the modulation of MMPs, RANKL,
635 and their physiological inhibitors. *Oral microbiology and immunology*, *21*(1), 12–20.
636 https://doi.org/10.1111/j.1399-302X.2005.00245.x
637
638 **Geng Y, Li L, Wang X, He F, Zhou Y, Yang M, & Xu Y. 2018.** Interleukin-10 polymorphisms
639 affect the key periodontal pathogens in Chinese periodontitis patients. *Scientific reports*, *8*(1),
640 9068. https://doi.org/10.1038/s41598-018-26236-4
641
642 **Gölz L, Memmert S, Rath-Deschner B, Jäger A, Appel T, Baumgarten G, Götz W, & Frede**
643 **S. 2014.** LPS from *P. gingivalis* and hypoxia increases oxidative stress in periodontal ligament
644 fibroblasts and contributes to periodontitis. *Mediators of inflammation*, *2014*, 986264.
645 https://doi.org/10.1155/2014/986264
646
647 **Gomes F I, Aragão M G, Barbosa F C, Bezerra MM, de Paulo Teixeira Pinto V, & Chaves**
648 **H V. 2016.** Inflammatory Cytokines Interleukin-1β and Tumour Necrosis Factor-α Novel
649 Biomarkers for the Detection of Periodontal Diseases: a Literature Review. *Journal of oral &*
650 *maxillofacial research*, *7*(2), e2. https://doi.org/10.5037/jomr.2016.7202
651
652 **Gorąca A, Huk-Kolega H, Kleniewska P, Piechota-Polańczyk A, & Skibska B. 2013.** Effects
653 of lipoic acid on spleen oxidative stress after LPS administration. *Pharmacological reports : PR*,
654 *65*(1), 179–186. https://doi.org/10.1016/s1734-1140(13)70976-9
655
656 **Gorr S U. 2009.** Antimicrobial peptides of the oral cavity. *Periodontology 2000*, *51*, 152–180.
657 https://doi.org/10.1111/j.1600-0757.2009.00310.x
658
659 **Gorr S U. 2012**. Antimicrobial peptides in periodontal innate defense. *Frontiers of oral*
660 *biology*, *15*, 84–98. https://doi.org/10.1159/000329673
661
662 **Graves D T, Oskoui M, Volejnikova S, Naguib G, Cai S, Desta T, Kakouras A, & Jiang Y.**
663 **2001.** Tumor necrosis factor modulates fibroblast apoptosis, PMN recruitment, and osteoclast
664 formation in response to *P. gingivalis* infection. *Journal of dental research*, *80*(10), 1875–1879.
665 https://doi.org/10.1177/00220345010800100301
666
667 **Gren S T, Janciauskiene S, Sandeep S, Jonigk D, Kvist P H, Gerwien J G, Håkansson K, &**
668 **Grip O. 2016.** The protease inhibitor cystatin C down-regulates the release of IL-β and TNF-α in
669 lipopolysaccharide activated monocytes. *Journal of leukocyte biology*, *100*(4), 811–822.
670 https://doi.org/10.1189/jlb.5A0415-174R
671
672 **Greer A, Zenobia C, & Darveau R P. 2013.** Defensins and LL-37: a review of function in the
673 gingival epithelium. *Periodontology 2000*, *63*(1), 67–79. https://doi.org/10.1111/prd.12028
674
675 **Gutiérrez-Venegas G, Maldonado-Frías S, Ontiveros-Granados A, & Kawasaki-Cárdenas**
676 **P. 2005.** Role of p38 in nitric oxide synthase and cyclooxygenase expression, and nitric oxide and

Código de campo cambiado

Código de campo cambiado

PGE2 synthesis in human gingival fibroblasts stimulated with lipopolysaccharides. *Life sciences*, *77*(1), 60–73. https://doi.org/10.1016/j.lfs.2004.12.015

Guo Y, Nguyen K A, & Potempa J. 2010 Dichotomy of gingipains action as virulence factors: from cleaving substrates with the precision of a surgeon's knife to a meat chopper-like brutal degradation of proteins. Periodontology 2000 54(1) 15–44. https://doi.org/10.1111/j.1600-0757.2010.00377.x

Hariyani N, Nur HA, Al-Junaid M, Fadhila O, Indah BT. 2021. Mouse periodontitis models using whole Poprhyromonas gingivalis bacteria induction. *Saudi Dental Journal*. https://doi.org/10.1016/j.sdentj.2021.08.001

Henry L G, McKenzie R M, Robles A, & Fletcher H M (2012). Oxidative stress resistance in *Porphyromonas gingivalis*. *Future microbiology*, *7*(4), 497–512. https://doi.org/10.2217/fmb.12.17

Herath T, Darveau R P, Seneviratne CJ, Wang C Y, Wang Y, & Jin L. 2016. Heterogeneous *Porphyromonas gingivalis* LPS modulates immuno-inflammatory response, antioxidant defense and cytoskeletal dynamics in human gingival fibroblasts. *Scientific reports*, *6*, 29829. https://doi.org/10.1038/srep29829

How KY, Song K P, & Chan KG. 2016. *Porphyromonas gingivalis*: An Overview of Periodontopathic Pathogen below the Gum Line. *Frontiers in microbiology*, *7*, 53. https://doi.org/10.3389/fmicb.2016.00053

Imamura T. 2003. The role of gingipains in the pathogenesis of periodontal disease. *Journal of . Periodontology*, 74 (1), 111- 118, https://doi.org/10.1902/jop.2003.74.1.111

Imatani T, Kato T, Minaguchi K, & Okuda K. 2000. Histatin 5 inhibits inflammatory cytokine induction from human gingival fibroblasts by *Porphyromonas gingivalis*. *Oral microbiology and immunology*, *15*(6), 378–382. https://doi.org/10.1034/j.1399-302x.2000.150607.x

Jadaun G P, Agarwal C, Sharma H, Ahmed Z, Upadhyay P, Faujdar J, Gupta A K, Das R., Gupta P, Chauhan D S, Sharma V D, & Katoch V M. 2007. Determination of ethambutol MICs for Mycobacterium tuberculosis and Mycobacterium avium isolates by resazurin microtitre assay. *The Journal of antimicrobial chemotherapy*, *60*(1), 152–155. https://doi.org/10.1093/jac/dkm117

Jia L, Han N, Du J, Guo L, Luo Z, & Liu Y. (2019). Pathogenesis of Important Virulence Factors of *Porphyromonas gingivalis* via Toll-Like Receptors. *Frontiers in cellular and infection microbiology*, *9*, 262. https://doi.org/10.3389/fcimb.2019.00262

Kanzaki H, Wada S, Narimiya T, Yamaguchi Y, Katsumata Y, Itohiya K, Fukaya S, Miyamoto Y and Nakamura Y. 2017. Pathways that Regulate ROS Scavenging Enzymes, and Their Role in Defense Against Tissue Destruction in Periodontitis. Front. Physiol. 8:351. doi: 10.3389/fphys.2017.00351

Código de campo cambiado

Kirkwood KL, Cirelli JA, Rogers JE, & Giannobile W V. 2007. Novel host response therapeutic approaches to treat periodontal diseases. *Periodontology 2000*, *43*, 294–315. https://doi.org/10.1111/j.1600-0757.2006.00166.x

Kobayashi-Sakamoto M, Isogai E, & Hirose K. 2003. *Porphyromonas gingivalis* modulates the production of interleukin 8 and monocyte chemotactic protein 1 in human vascular endothelial cells. *Current microbiology*, *46*(2), 109–114. https://doi.org/10.1007/s00284-002-3782-x

Könönen E, Gursoy M, & Gursoy U K. 2019. Periodontitis: A Multifaceted Disease of Tooth-Supporting Tissues. *Journal of clinical medicine*, *8*(8), 1135. https://doi.org/10.3390/jcm8081135

Kratzer E, Tian Y, Sarich N, Wu T, Meliton A, Leff A, & Birukova A A. 2012. Oxidative stress contributes to lung injury and barrier dysfunction via microtubule destabilization. *American journal of respiratory cell and molecular biology*, *47*(5), 688–697. https://doi.org/10.1165/rcmb.2012-0161OC

Latz E, Xiao T S, & Stutz A. 2013. Activation and regulation of the inflammasomes. *Nature reviews. Immunology*, *13*(6), 397–411. https://doi.org/10.1038/nri3452

Lee Ik, Lee Mj & Jang HS. 2013. The interrelationship between human gingival fibroblast differentiation and cultivating time. *Tissue Eng Regen Med* **10,** 60–64 (2013). https://doi.org/10.1007/s13770-013-0371-y

Lee S M, Son K N, Shah D, Ali M, Balasubramaniam A, Shukla D, & Aakalu V K 2021. Histatin-1 Attenuates LPS-Induced Inflammatory Signaling in RAW264.7 Macrophages. *International journal of molecular sciences*, *22*(15), 7856. https://doi.org/10.3390/ijms22157856

Liang W, & Diana J. 2020. The Dual Role of Antimicrobial Peptides in Autoimmunity. *Frontiers in immunology*, *11*, 2077. https://doi.org/10.3389/fimmu.2020.02077

Liu J, Wang Y, Shi Q, Wang X, Zou P, Zheng M, & Luan Q. 2022. Mitochondrial DNA Efflux Maintained in Gingival Fibroblasts of Patients with Periodontitis through ROS/mPTP Pathway. *Oxidative medicine and cellular longevity*, *2022*, 1000213. https://doi.org/10.1155/2022/1000213

McFarland J. 1907 Nephelometer: An Instrument for Estimating the Number of Bacteria in Suspensions Used for Calculating the Opsonic Index and for Vaccines. Journal of the American Medical Association, 14, 1176-1178. http://dx.doi.org/10.1001/jama.1907.25320140022001f

Mei F, Xie M, Huang X, Long Y, Lu X, Wang X, & Chen L. 2020. *Porphyromonas gingivalis* and Its Systemic Impact: Current Status. *Pathogens (Basel, Switzerland)*, *9*(11), 944. https://doi.org/10.3390/pathogens9110944

Melo E S, Barbeiro H V, Ariga S, Goloubkova T, Curi R, Velasco I T, Vasconcelos D, & Soriano F G. 2010. Immune cells and oxidative stress in the endotoxin tolerance mouse model.

Código de campo cambiado

Dio formato

Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas, *43*(1), 57–67. https://doi.org/10.1590/s0100-879x2009007500027

Mendez KN, Hoare A, Soto C, Bugueño I, Olivera M, Meneses C, Pérez-Donoso JM, Castro-Nallar E & Bravo D . 2019. Variability in Genomic and Virulent Properties of *Porphyromonas gingivalis* Strains Isolated From Healthy and Severe Chronic Periodontitis Individuals. *Frontiers in cellular and infection microbiology*, *9*, 246. https://doi.org/10.3389/fcimb.2019.00246

Maruyama T, Tomofuji T, Endo Y, Irie K, Azuma T, Ekuni D, Tamaki N, Yamamoto T, & Morita M. 2011. Supplementation of green tea catechins in dentifrices suppresses gingival oxidative stress and periodontal inflammation. *Archives of oral biology*, *56*(1), 48–53. https://doi.org/10.1016/j.archoralbio.2010.08.015

Menu P, & Vince J E. 2011. The NLRP3 inflammasome in health and disease: the good, the bad and the ugly. *Clinical and experimental immunology*, *166*(1), 1–15. https://doi.org/10.1111/j.1365-2249.2011.04440.x

Mickels N, McManus C, Massaro J, Friden P, Braman V, D'Agostino R, Oppenheim F, Warbington M, Dibart S, Van Dyke T. 2001. Clinical and microbial evaluation of a histatin-containing mouthrinse in humans with experimental gingivitis. J Clin Periodontol. May;28(5):404-10. doi: 10.1034/j.1600-051x.2001.028005404.x

Nogueira T P, Gonçalves BF, Gabriela S D, Hebling J, de Souza CC. 2016. Functional Differences In Gingival Fibroblasts Obtained from Young and Elderly Individuals. Braz. Dent. J. 27 (5) https://doi.org/10.1590/0103-6440201600993

Pacher P, Beckman J S, & Liaudet L. 2007. Nitric oxide and peroxynitrite in health and disease. *Physiological reviews*, *87*(1), 315–424. https://doi.org/10.1152/physrev.00029.2006

Palm E, Khalaf H, & Bengtsson T. 2013. *Porphyromonas gingivalis* downregulates the immune response of fibroblasts. *BMC microbiology*, *13*, 155. https://doi.org/10.1186/1471-2180-13-155

Palm E, Khalaf H, & Bengtsson T. 2015. Suppression of inflammatory responses of human gingival fibroblasts by gingipains from *Porphyromonas gingivalis*. *Molecular oral microbiology*, *30*(1), 74–85. https://doi.org/10.1111/omi.12073

Pan W, Wang Q, & Chen Q. 2019. The cytokine network involved in the host immune response to periodontitis. *International journal of oral science*, *11*(3), 30. https://doi.org/10.1038/s41368-019-0064-z

Rafiei M, Kiani F, Sayehmiri F, Sayehmiri K, Sheikhi A & Zamanian Azodi M. 2017. Study of *Porphyromonas gingivalis* in periodontal diseases: A systematic review and meta-analysis. *Medical journal of the Islamic Republic of Iran*, *31*, 62. https://doi.org/10.18869/mjiri.31.62

Código de campo cambiado

Ramadan DE, Hariyani N, Indrawati R, Ridwan R D, & Diyatri I. 2020. Cytokines and Chemokines in Periodontitis. *European journal of dentistry*, *14*(3), 483–495. https://doi.org/10.1055/s-0040-1712718

Sanikidze T V, Tkhilava N G, Papava M B, Datunashvili I V, Gongadze M T, Gamrekelashvili D D, & Bakhutashvili V I. 2006. Role of free nitrogen and oxygen radicals in the pathogenesis of lipopolysaccharide-induced endotoxemia. *Bulletin of experimental biology and medicine*, *141*(2), 211–215. https://doi.org/10.1007/s10517-006-0130-3

Shamsi A, & Bano B. 2017. Journey of cystatins from being mere thiol protease inhibitors to at heart of many pathological conditions. *International journal of biological macromolecules*, *102*, 674–693. https://doi.org/10.1016/j.ijbiomac.2017.04.071

Smith PC, Martínez C, Martínez J, & McCulloch C A. 2019. Role of Fibroblast Populations in Periodontal Wound Healing and Tissue Remodeling. *Frontiers in physiology*, *10*, 270. https://doi.org/10.3389/fphys.2019.00270

Sosroseno W, Bird P S & Seymour G J. 2009. Nitric oxide production by a human osteoblast cell line stimulated with *Aggregatibacter actinomycetemcomitans* lipopolysaccharide. Oral Microbiol Immunol 24 (1) 50–55 doi: 10.1111/j.1399-302X.2008.00475.x.

Song H K, Noh E , KimJ M, You Y O, Kwon K B, & Lee Y R. 2021. *Evodiae fructus* Extract Inhibits Interleukin-1β-Induced MMP-1, MMP-3, and Inflammatory Cytokine Expression by Suppressing the Activation of MAPK and STAT-3 in Human Gingival Fibroblasts In Vitro. *Evidence-based complementary and alternative medicine : eCAM*, *2021*, 5858393. https://doi.org/10.1155/2021/5858393

Sun W, Wu J, Lin L, Huang Y, Chen Q, & Ji Y. 2010. *Porphyromonas gingivali*s stimulates the release of nitric oxide by inducing expression of inducible nitric oxide synthases and inhibiting endothelial nitric oxide synthases. *Journal of periodontal research*, *45*(3), 381–388. https://doi.org/10.1111/j.1600-0765.2009.01249.x

Sun T, Wang F, Pan W, Wu Q, Wang J, & Dai J. 2018. An Immunosuppressive Tick Salivary Gland Protein DsCystatin Interferes With Toll-Like Receptor Signaling by Downregulating TRAF6. *Frontiers in immunology*, *9*, 1245. https://doi.org/10.3389/fimmu.2018.01245

Sun L, Girnary M, Wang L, Jiao Y, Zeng E, Mercer K, Zhang J, Marchesan J T, Yu N, Moss K, Lei Y L, Offenbacher S, & Zhang S. 2020. IL-10 Dampens an IL-17-Mediated Periodontitis-Associated Inflammatory Network. *Journal of immunology (Baltimore, Md. : 1950)*, *204*(8), 2177–2191. https://doi.org/10.4049/jimmunol.1900532

Tomofuji T, Azuma T, Kusano H, Sanbe T, Ekuni D, Tamaki N, Yamamoto T, & Watanabe T. 2006. Oxidative damage of periodontal tissue in the rat periodontitis model: effects of a high-cholesterol diet. *FEBS letters*, *580* (15), 3601–3604. https://doi.org/10.1016/j.febslet.2006.05.041

Torbjörn B, Atika K, Hazem Khalaf. 2015. Secreted gingipains from *Porphyromonas gingivalis* colonies exert potent immunomodulatory effects on human gingival fibroblasts. Microbiological Research. 178 (2015) :18-26. http://dx.doi.org/10.1016/j.micres.2015.05.00

Uehara A, Naito M, Imamura T, Potempa J, Travis J, Nakayama K, and Takada, H. 2008. Dual regulation of interleukin-8 production in human oral epithelial cells upon stimulation with gingipains from *Porphyromonas gingivalis.* J. Med. Microbiol. 57, 500–507

van Winkelhoff, AJ, Loos BG, van der Reijden WA, van der Velden U. 2002 ,*Porphyromonas gingivalis*, *Bacteroides forsythus* and other putative periodontal pathogens in subjects with and without periodontal destruction. *Journal of Clinical Periodontology.* 29(11):1023-1028. https://doi.org/10.1034/j.1600-051X.2002.291107.x

van Wyk S G, Kunert K J, Cullis C A, Pillay P, Makgopa M E, Schlüter U, & Vorster B J .2016. Review: The future of cystatin engineering. *Plant science : an international journal of experimental plant biology*, *246*, 119–127. https://doi.org/10.1016/j.plantsci.2016.02.016

Vray B, Hartmann S, & Hoebeke J. 2002. Immunomodulatory properties of cystatins. *Cellular and molecular life sciences : CMLS*, *59*(9), 1503–1512. https://doi.org/10.1007/s00018-002-8525-4

Wang H, Zhou H, Duan X, Jotwani R, Vuddaraju H, Liang S, Scott DA, & Lamont R J. 2014. *Porphyromonas gingivalis*-induced reactive oxygen species activate JAK2 and regulate production of inflammatory cytokines through c-Jun. *Infection and immunity*, *82*(10), 4118–4126. https://doi.org/10.1128/IAI.02000-14

Wang H, Ai L, Zhang Y, Cheng J, Yu H, Li C, Zhang D, Pan Y, Lin L. 2018. The Effects of Antimicrobial Peptide Nal-P-113 on Inhibiting Periodontal Pathogens and Improving Periodontal Status. Biomed Res Int.15; 2018:1805793. doi: 10.1155/2018/1805793

Wang Y, Huang X, & He F 2019. Mechanism and role of nitric oxide signaling in periodontitis,.Exp Ther Med 18, 1503-1512

Xie H, Wu L, Chen X, Gao S, Li H, Yuan Y, Liang J, Wang X, Wang S, Xu C, Chu L, Zhan B, Zhou R & Yang X. 2021. *Schistosoma japonicum* Cystatin Alleviates Sepsis Through Activating Regulatory Macrophages. *Frontiers in cellular and infection microbiology*, *11*, 617461. https://doi.org/10.3389/fcimb.2021.617461

Zhu C, Zhao Y, Wu X, Qiang C, Liu J, Shi J, Gou J, Pei D, Li A. 2020. The therapeutic role of baicalein in combating experimental periodontitis with diabetes via Nrf2 antioxidant signaling pathway. J Periodontal Res. 55(3):381-391. doi: 10.1111/jre.12722.

Zumft W. G. (2002). Nitric oxide signaling and NO dependent transcriptional control in bacterial denitrification by members of the FNR-CRP regulator family. *Journal of molecular microbiology and biotechnology*, *4*(3), 277–286.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding Ana María Fernández Presas
This work was supported by grant # IN218419, from PAPITT, DGAPA, UNAM, Mexico City, and partially by Universidad Anahuac México Campus Norte . Blanca Esther Blancas-Luciano is supported by CONACYT grant # 424031 for her doctoral studies. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures The following grant information was disclosed by the authors:
PAPITT, DGAPA, UNAM, Mexico City: #IN218419.
CONACYT: # 424031

Competing Interests The authors declare there are no competing interests.

Author Contributions
Blanca Blancas, Jaime Zamora, Adriana Remigio, and Delgado Dominguez participate in the conceptualization, study design, conducted all the experiments, Data curation, and reviewed drafts of the paper
r, Leyva-Huerta ER, Portilla-Robertson J, participate in the conceptualization, data curation and reviewed drafts of the paper.
I. Becker was involved in the concept of the study, Supervision, Visualization, critically revised the manuscript, and approved the final draft,
A.M Fernández participated in Conceptualization, study design, conducted experiments, supervision, visualization interpreted the data, drafted the manuscript, approved the final draft. and acquired the funding.

Data Availability

The following information was supplied regarding data availability: