



**UNESP - Universidade Estadual Paulista
“Júlio de Mesquita Filho”
Faculdade de Odontologia de Araraquara**



Natalia Da Ponte Leguizamón

**Phytocystatin *CsinCPI-2* prevents bone loss in ligature-induced periodontitis by
inhibition of inflammation and osteoclastogenesis**

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Thesis presented to the Graduate Program in Dentistry, area of Periodontics, School of Dentistry at Araraquara, Sao Paulo State University (UNESP), as a prerequisite to obtain the degree of PhD in Dentistry.

Tutor: Prof. Dr. Joni Augusto Cirelli

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Natalia Da Ponte Leguizamón

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Examining Board

Thesis presented to obtain the degree of PhD in Dentistry

President and Tutor: Prof. Dr. Joni Augusto Cirelli

2nd Examiner: Dr. Renato Corrêa Viana Casarin

3rd Examiner: Dr. Mauro Pedrine Santamaria

4th Examiner: Dr Willian Fernando Zambuzzi

Araraquara, June 29, 2021

CURRICULAR DATA

Natalia Da Ponte Leguizamón

Birth:	July 24, 1986 Asunción, Paraguay
Affiliation:	Maria Mercedes Leguizamón Zaván Jorge Luis Da Ponte Gonzalez
2005-2012:	Dentist degree School of Dentistry National University of Asunción – UNA Paraguay
2011-2013:	Specialization Course in Oral Rehabilitation School of Dentistry National University of Asunción – UNA Paraguay
2014:	Advanced Training Course School of Dentistry National University of Asunción – UNA Paraguay
2015-2017:	Postgraduate course in Periodontics Master level School of Dentistry, Araraquara São Paulo State University – UNESP Brazil
2017-2021:	Postgraduate course in Periodontics PhD level School of Dentistry, Araraquara São Paulo State University – UNESP Brazil
2019-2021:	Specialization Course in Implant Dentistry School of Dentistry APCD Sao Paulo State, Brazil
2019-2020:	Internship in the Department of Periodontology and Operative Dentistry University Medical Center of the Johannes Gutenberg University Mainz, Germany

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"It's not how much we give, but how much love we put into giving"

* Mother Teresa. <https://www.goodreads.com>.

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ABSTRACT

Periodontal disease (PD) is a polymicrobial chronic inflammatory condition of the supporting tissues around the teeth, leading to the destruction of surrounding connective tissue. During the progression of PD, osteoclasts play a crucial role in the resorption of alveolar bone that eventually leads to the loss of teeth if the PD is left untreated. Therefore, the development of anti-resorptive therapies targeting bone-resorbing cells will significantly benefit the treatment of PD. Here, we demonstrate the inhibitory effect of CsinCPI-2, a novel cysteine peptidase inhibitor from the orange tree, on periodontitis-induced inflammation, alveolar bone loss and osteoclast differentiation. Using the ligature-induced periodontitis model in mice, we demonstrate that treatment with CsinCPI-2 (0.8 µg/g of body weight) significantly reduced inflammatory cell infiltrate in the connective tissue and prevents the loss of alveolar bone mass (BV/TV) caused by PD, effects associated with diminished numbers of TRAP-positive multinucleated cells. Furthermore, CsinCPI-2 significantly down-regulated the numbers of inflammatory cells expressing CD3, CD45 MAC387 and IL-1β. *In vitro*, CsinCPI-2 inhibited RANKL-induced TRAP⁺ multinucleated osteoclast formation in mouse bone marrow macrophage (BMM) cultures in a concentration-dependent manner. This effect was not due to cytotoxicity, as demonstrated by the MTT assay. CsinCPI-2 inhibited RANKL-induced mRNA expression of *Acp5*, *Calcr* and *Ctsk*, as well as the RANKL-induced up-regulation of *Nfatc1*, a crucial transcription factor for osteoclast differentiation. Our findings demonstrate that CsinCPI-2 prevents bone loss induced by PD by controlling the inflammatory process and acting directly on osteoclastogenesis, suggesting an interesting potential for CsinCPI-2 in the strategy for PD treatment.

Key-words: Bone. Periodontal diseases. Periodontitis. Osteoclast. Inflammation.

Da Ponte Leguizamón N. Fitocistatina CsinCPI-2 evita a perda óssea na periodontite induzida por ligadura pela inibição da inflamação e osteoclastogênese [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2021.

RESUMO

A doença periodontal (DP) é uma condição inflamatória polimicrobiana crônica dos tecidos de suporte ao redor dos dentes, que levam à destruição do tecido conjuntivo circundante. Durante a progressão da DP, os osteoclastos desempenham um papel crucial na reabsorção do osso alveolar que, eventualmente, leva à perda de dentes caso a DP não seja tratada. Portanto, o desenvolvimento de terapias antirreabsortivas direcionadas às células responsáveis pela reabsorção óssea irão beneficiar significativamente o tratamento da DP. Aqui demonstramos o efeito inibitório da CsinCPI-2, um novo inibidor da cisteína peptidase da laranjeira na inflamação induzida por periodontite, perda óssea alveolar e diferenciação de osteoclastos. Usando o modelo de periodontite induzida por ligadura em camundongos, demonstramos que o tratamento com CsinCPI-2 (0,8 µg / g de peso corporal) reduziu significativamente o infiltrado de células inflamatórias no tecido conjuntivo e preveniu a perda de massa óssea alveolar (BV / TV) causada pela DP, efeitos estes associados a números diminuídos de células multinucleadas TRAP-positivas. Além disso, a CsinCPI-2 regulou negativamente o número de células inflamatórias que expressam CD3, CD45 MAC387 e IL-1β. In vitro, a CsinCPI-2 inibiu a formação de osteoclastos multinucleados TRAP +, induzida por RANKL em culturas de macrófagos da medula óssea (BMM) de camundongo de uma maneira dependente da concentração. Este efeito não foi devido à citotoxicidade, conforme demonstrado pelo ensaio MTT. CsinCPI-2 inibiu a expressão de mRNA induzida por RANKL de Acp5, Calcr e Ctsk, bem como a regulação positiva induzida por RANKL de Nfatc1, um fator de transcrição crucial para a diferenciação de osteoclastos. Nossos achados demonstram que a CsinCPI-2 previne a perda óssea induzida pela DP por meio do controle do processo inflamatório e ação direta na osteoclastogênese, sugerindo um potencial interessante para a CsinCPI-2 na estratégia de tratamento da DP.

Palavras-chave: Osso. Doença periodontal. Periodontite. Osteoclasto. Inflamação.

ABBREVIATIONS AND ACRONYMS

ABC	Avidin-Biotin Peroxidase
ABC	Alveolar bone crest
ABL	Alveolar bone loss
Acp5	Acid Phosphatase 5
BMD	Bone mineral density
BMM	Bone marrow macrophages
BSA	Bovine serum albumin
BV	Bone volume
BV/TV	Bone volume fraction
CaHA	Calcium Hydroxyapatite
Calcr	Calcitonin receptor
CD3	Marker of T cells
CD45	Marker of leukocyte
CEJ	Cement-enamel junction
CP	Cysteine peptidases
CsinCPI-2	Citrus sinensis derived inhibitor
CTL	Control
Ctsk	Cathepsin K
CysC	Cystatin C
H&E	Hematoxylin and Eosin
Il-1 β	Interleukin 1 Beta
IL-6	Interleukin 6
IL-8	Interleukin 8
Irf8	Interferon Regulatory factor 8
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor (M-CSF)
M-PER	Mammalian Protein Extraction Reagent
MAC387	Marker of macrophages
Micro-CT	Micro-computed tomography
MTT	Methyl tetrazolium
MuOCLs	Multinucleated osteoclasts
Nf- κ B	Receptor activator of nuclear factor- κ B
NFATc1	Cytoplasmic 1
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PD	Periodontal disease
PMNs	Polymorphonuclear leukocytes
RANKL	Receptor activator of nuclear factor- κ B ligand
RAW264.7	Mouse macrophage cell line
ROI	Region of interest
RT-qPCR	Real-Time Polymerase Chain Reaction
TBS	Tris-buffered saline
TBST	TBS with 0.05% Tween-20
TNF- α	Tumor Necrosis Factor Alpha
Tnfrsf11a	TNF Receptor Superfamily Member 11a
TRAP	Tartrate-resistant acid phosphatase
TV	Tissue volume

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1 INTRODUCTION

Periodontal disease (PD) is a chronic inflammatory condition of the supporting tissues around the teeth, mainly caused by a microbial dysbiosis and dental biofilm accumulation on tooth surfaces¹⁻². The host immune response plays an essential role in the progression and severity of the disease². If PD is left untreated, exacerbation of the host immune response as a consequence of the microbial injury leads to destruction of mineralized and non-mineralized tissues, including gingiva, periodontal ligament, and alveolar bone³.

The conventional therapy of PD is the mechanical disruption and removal of bacterial biofilms on tooth surfaces and adjacent soft tissue, but many studies have suggested the use of adjunctive host-modulating drugs as a new strategy in the management of PD⁴.

In this context, there is increasing interest in alternative medicine and natural products for the treatment of PD with a number of pre-clinical studies in the literature using naturally-occurring chemical compounds with positive effects on ameliorating the inflammatory condition and PD induced-bone loss⁵⁻⁹. Recently, our group identified several phytocystatins derived from different plants with robust cysteine peptidase inhibition activity¹⁰⁻¹⁴. Since the endogenous cysteine proteinase inhibitor cystatin C and the fungal cysteine proteinase inhibitor E-64 inhibit both RANKL and LPS-induced osteoclast formation¹⁵⁻¹⁶, we hypothesized that phytocystatins may also block osteoclastogenesis. Among the phytocystatins, we selected a *Citrus sinensis* (orange) derived inhibitor (CsinCPI-2) due to its ability to inhibit not only the enzymatic activity of cathepsin K but also the up-regulation of Ctsk, Il-1 β and TNF- α mRNA expression induced by *P. gingivalis* in the macrophage cell line RAW264.7¹⁰.

2 OBJECTIVES

The aim of this study was to evaluate the preventive effect of *Csin*CPI-2 in the periodontal destruction in an experimental periodontitis model in mice. In addition, the direct effect of *Csin*CPI-2 on osteoclastogenesis was evaluated in vitro.

3 LITERATURE REVIEW

3.1 Periodontal Disease

Periodontal disease (PD) is a multi-factorial disorder that is initiated by a microbial dysbiosis¹⁷⁻¹⁹, characterized by a disruption of the homeostatic balance between the host and the bacterial population²⁰. It is the major disease that affects the oral cavity²⁰ and this disease is a widespread health problem estimated to affect 47.2% of the USA adult population²¹.

PD affects the structures that support the teeth and can lead to alveolar bone loss and tooth exfoliation¹⁷. As in any inflammatory disease, the immune-inflammatory response of the host is responsible for the progression of the pathological process.²²⁻²³.

The inflammation is initiated by bacteria, but the host response to them is considered the main cause of the periodontium destruction²⁴. This destruction is the result of the interaction between the innate and acquired immune responses to the bacteria²⁵. Failure to eradicate the invading pathogens will result in a continued state of inflammation where inflammatory and resident cells release different factors that contribute to progression of the disease and lead to tissue destruction by producing degradative enzymes, apoptotic factors, and bone-resorbing mediators²²⁻²⁶⁻²⁷.

Inflammatory cells such as polymorphonuclear leukocytes (PMNs), lymphocytes, and macrophages participate in the infiltration of the periodontium. Lymphocytes, the prominent leukocytes, secrete a number of molecules that directly or indirectly induce or inhibit osteoclast formation²⁷. Macrophages are essential for the innate and acquired response to intracellular infection and are the key link between the immune system and bone resorption²⁷. Macrophages are capable of differentiating to osteoclast in response to Tumor Necrosis Factor alpha (TNF- α) in the presence of receptor activator of nuclear factor- κ B (Nf- κ B) ligand (RANKL)²⁷.

3.2 Mediators in Periodontal Disease

The host periodontal tissues respond to the bacterial biofilm with a complex interaction between numbers of cell types and mediators⁴. Among these, cytokine, chemokines, and metalloproteinases are pro-inflammatory mediators that are increased in periodontal tissues and gingival crevicular fluid in PD¹⁹.

Pro-inflammatory cytokines like TNF- α , interleukin-1 β (IL-1 β), IL-8, and IL-6 are the first mediators that the host releases to initiate and sustain the process¹⁷⁻¹⁸⁻²⁸. These cytokines are involved in the induction of adhesion molecules, promoting leukocyte migration, stimulating the matrix metalloproteinase, and enhancing differentiation of osteoclasts on alveolar bone, thus leading to bone resorption²³⁻²⁶⁻²⁹.

IL-1 and TNF- α stimulate bone resorption by increasing osteoclast formation and promoting RANKL expression by marrow stromal cells and osteoblasts²⁵⁻³⁰.

Balancing the immune response in periodontitis has been taken into consideration in the literature as an alternative treatment of the disease. Some modulatory agents in inflammation were considered useful candidates to prevent the destruction of periodontal tissues. Treatments using IL-1 and/or TNF- α antagonists, or different inhibitors with anti-inflammatory activity have shown significant reduction of inflammation, connective tissue attachment loss, and bone resorption in experimental periodontitis in animals¹⁷⁻²²⁻²⁸⁻³¹.

3.3 Osteoclastogenesis Mechanisms

A hallmark of PD is the resorption of the alveolar bone surrounding the tooth. Bone tissues integrity depends on maintaining a balance between bone resorption and bone formation by the activity of osteoclasts and osteoblasts¹⁶⁻³².

Osteoclasts, multinucleated giant cells derived from bone marrow hematopoietic precursors, are the only cells in the body capable of resorbing bone³³. Precursor cells differentiate into osteoclasts in response to macrophage colony-stimulating factor (M-CSF) and RANKL. M-CSF is crucial for the proliferation and survival of monocytes and for priming macrophage/monocytes precursors towards the osteoclast lineage by stimulating the expression of RANK³⁴.

Osteoclastogenesis is produced by the NF- κ B mediated mechanism, resulting in the activation of the RANK receptor expressed on osteoclast progenitor cells leading to osteoclast lineage differentiation¹⁵. Since the proliferation and survival of the osteoclast progenitor cell is the activation of the ligand M-CSF to the receptor Csf-1, this leads to activating different osteoclast specific marker genes like Tartrate-resistant acid phosphatase (TRAP), Calcitonin receptor (CalcR), Acid Phosphatase 5 (Acp5), and Cathepsin K (Ctsk), that are important for osteoclast differentiation, maturation, and function.

During periodontitis, RANKL levels are increased both by signals triggered by pathogen-associated molecular patterns (PAMPs) binding to pathogen recognition receptors expressed by the host cells, and by signals from host-derived pro-inflammatory cytokines produced in response to inflammation³⁵. Apart from indirectly stimulating osteoclastogenesis through RANKL regulation, PAMPs and cytokines can also directly interfere in osteoclast differentiation and function by binding to receptors expressed by these cells and by their precursors³⁶⁻³⁷. Because elevated osteoclast formation and/or over-activation of its activity are the major underlying causes of alveolar bone loss and loosened teeth³⁸, targeting osteoclastogenesis may be an interesting approach to modulate periodontitis-induced bone resorption.

3.4 Cystatins

Cystatins are endogenous inhibitors of cysteine peptidases (CP) and they are present in tissues and fluids of the human body¹⁰⁻³⁹. They are tight-binding competitive inhibitors that block the proteolytic activity of CP⁴⁰. The cystatin superfamily inhibits family C1 of cysteine proteinases or papain superfamily, acting as pseudo-substrates blocking the active site of cysteine proteinases¹²⁻⁴⁰. The cystatin superfamily is subdivided into four families, three of mammal origin that are: family I or stefins, family II or cystatins (including salivary cystatins) and family III or kininogens; and one that is present in plants, named phytocystatins¹²⁻⁴¹.

Henskens et al.³⁹, 1993 found an increased cystatin activity in periodontal patients compared to healthy patients. Different cystatins isoforms have been identified in human saliva and in acquired enamel pellicle, suggesting a possible role against microorganisms¹³.

Cystatin C (CysC) is the most extracellularly abundant type of cystatin and it is involved in osteoclast formation and bone resorption¹⁵⁻⁴². CysC shows inhibitory activity in osteoclast formation, an effect that has been associated with decreased mRNA and protein expression of tartrate-resistant acid phosphatase (TRAP), cathepsin k, and c-Fos and NFATc1 expressions, which are osteoclastogenic transcription factors¹⁵.

Phytocystatins are found naturally in plants. The first cystatin derived from sugar cane was characterized and named Canacystatin (CaneCPI-1)¹⁴. This protein showed inhibitory activity against human cathepsins B, K, L, and V as well as other cysteine peptidases⁴³. Since cystatins are classified according to structural differences, it is expected that each cystatin has specific inhibitory activity profiles against cysteine proteinases, such as cathepsins. Other cystatins derived from sugar cane were characterized according to their activities in CaneCPI-1, CaneCPI-2, CaneCPI-3,

CaneCPI-4 and CaneCPI-5¹³⁻⁴⁴. CaneCPI-4 showed inhibitory activity against human cathepsins B and L, and also prevented the invasion of breast cancer cells, melanoma growth, angiogenesis, and metastasis⁴⁴⁻⁴⁵. In inhibitory assays, CaneCPI-5 demonstrated that it is a potent inhibitor of human cathepsins B, K, and L¹³.

Alternative cystatins homologs are required because of the inaccessible cost of human recombinant cystatin. Considering that Citrus fruits are important to human nutrition and represent a high value crop in worldwide agriculture¹², a new phytocystatin derived from *Citrus Sinensis* was developed¹⁰. *Citrus Sinensis*, named CsinCPI-2, was obtained and cloned into the eukaryotic expression vector pET28a and was overexpressed in *E. coli* cells¹⁰. In a previous in vitro study, CsinCPI-2 has shown inhibitory activity against human cathepsin K and B by fluorometric assay. It has also shown inhibition of cathepsin K, B, and TNF- α in murine macrophage cells (RAW 264.7) stimulated with *P.gingivalis*¹⁰. In an in vivo study, CsinCPI-2 inhibited TNF- α production in blood plasma of rats after LPS stimulus¹⁰.

4 MATERIALS AND METHODS

4.1 Materials

Recombinant mouse M-CSF, recombinant human M-CSF, and recombinant mouse RANKL (Lys158-Asp316, cat. no. 462-TEC), were purchased from R&D Systems (Minneapolis, MN, USA). The kit for leukocyte tartrate-resistant acid phosphatase (TRAP)(387-A) staining and FITC-labeled phalloidin, were purchased from Sigma-Aldrich (St. Louis, MO, USA). α -Modification of minimum essential medium (α -MEM), and fetal bovine serum (FBS) were purchased from Life Technologies Corp. (Carlsbad, CA, USA). For in vitro experiments, oligonucleotide primers were purchased from Invitrogen (Carlsbad, CA, USA). RNAqueous®-Micro total RNA extraction Kit, High Capacity cDNA Reverse Transcription, and SyBr Universal PCR Master Mix were purchased from Applied Biosystems (Foster City, CA, USA). BCA protein assay kit was purchased from Pierce (Rockford, IL, USA). DAPI was purchased from Invitrogen. Anti-mouse Nfatc1 mouse monoclonal IgG1 (sc-7294; 7A6) and secondary antibody goat anti-mouse (SC-2005) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mammalian protein extraction reagent (M-PER) was purchased from Thermo Fisher (Waltham, MA, USA). ECL Plus Western Blotting Substrate and Methyl tetrazolium (MTT) assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Multi-well plates and Petri dishes were purchased from Corning (Corning, NY, USA). IHC technique was performed with the LSAB kit, using silanized slides and DAB staining from Dako (Glostrup, Denmark). Rodent Decloaker reagent (Biocare Medical; Concord, CA). 5% bovine serum albumin (BSA, Sigma). The primary antibodies were purchased from Abcam (Cambridge, UK). For in vivo experiments, Taqman assays were purchased from Applied Biosystems, RNeasy kit from Qiagen (Germantown, MD, USA), High

Capacity cDNA synthesis and Taqman Universal PCR Master Mix from Applied Biosystems (Cambridge, UK).

4.2 Methods

4.2.1 Expression and purification of recombinant CsinCPI-2 protein

The recombinant phytocystatin was produced as previously described¹⁰. Briefly, the cDNA coding *CsinCPI-2* was cloned in pET28a vector and expressed in *Escherichia coli* cells. Recombinant expression and purification were performed as described by¹⁴. *CsinCPI-2* protein was purified by affinity chromatography and dialyzed in phosphate-buffered saline with a pH of 7.4. Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

4.2.2 In vivo experiments

4.2.2.1 Animal care

The study protocol was approved (protocol # 31/2018 – APPENDIX A) by the Ethics Committee on Animal Usage (CEUA) of the School of Dentistry at Araraquara (Sao Paulo State University - UNESP, Brazil) in compliance with the applicable ethical guidelines and regulations of the International Guiding Principles for Biomedical Research Involving Animals. All recommended ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines were followed in this study⁴⁶.

4.2.2.2 Sample size calculation

The sample size for this study was calculated using the G*Power 3.1 software⁴⁷ and was based on data from a previously published study⁴⁸⁻⁴⁹. Inflammatory bone resorption of the alveolar bone (alveolar bone loss - ABL) was 05 (type II error) and a power of 80% (type I error) to detect a difference of approximately 10% in ABL between the two experimental groups. The effect size was determined as 2.91, and based on this finding,

each group should include 6 animals. Considering the possibility of unexpected death during the experiment, 8 animals were included in each experimental group and set as the main outcome. The effect size was calculated considering an alpha level of 0.

4.2.2.3 Study protocol

A total of 24 male C57BL/6J mice of 8 weeks of age and weighing approximately 25g received intraperitoneal (i.p.) injection with a volume of 200 μ L of vehicle or CsinCPI-2 (0.8 μ g/g) every day, during the entire experimental period (15 days). Mice were randomly (raffle method), divided into three experimental groups (n= 8/group), according to the ascribed treatment: the control group – animals received i.p. injection of PBS and were not ligated; PD group – animals received ligature around the first maxillary molar and received i.p. injections of PBS; and PD + CsinCPI-2 group - animals were ligated and treated with i.p. injection of 0.8 mg/g of CsinCPI-2. The dosage of CsinCPI-2 was based on a previous experiment recently published by our group¹⁰. All animals were kept in standard plastic cages separated by group under controlled environmental conditions (23°C $\pm$ 2°C, humidity and 12-hour dark/light cycle), and could freely access to standard rodent chow and tap water.

During the experiment, only one person was aware of the treatment group allocation. Each analysis performed (n=8/group) was conducted by a single experienced and trained examiner blind to the experimental groups.

All animals were overseen daily throughout the entire experimental period to evaluate any signs of adverse effects that the systemic administration of CsinCPI-2 might have caused. None of the mice developed any signs or symptoms of adverse effects caused by daily administration of the compound. No animals were lost during the experimental period.

4.2.2.4 Ligature-induced bone loss

Animals were anesthetized with a combination of ketamine hydrochloride (0.08 mL/100g body weight) and xylazine hydrochloride 2% (0.04 mL/100g). The upper first molars bilaterally received a silk 5.0 thread ligature tied around the cervical portion of the tooth, as described previously⁵⁰. Immediately after ligature placement, mice received either PBS or CsinCPI-2 every day, according to the experimental groups. No ligatures were lost during the entire experimental period.

Fifteen days after ligature application, all animals were euthanized by an overdose of anesthetic. Then, the hemi-maxillae were carefully resected and were fixed in 4% paraformaldehyde for 48 h and stored in 70% ethanol until scanned on a micro-CT imaging system. After scanning, the hemi-maxillae with intact soft tissue were decalcified in EDTA (0.5 M, pH 8.0) for five weeks. The reduced specimens (consisting of a section containing all the three molars and their surrounding alveolar process and soft tissues) were included in paraffin blocks. Semi-serial sections of 5 µm were obtained in the mesiodistal orientation (sagittal plane). Each of the analyses was conducted by a single experienced and trained examiner, who was blind to the experimental groups.

4.2.2.5 Micro-computed tomography (Micro-CT)

The maxillae were carefully dissected, fixed in 4% paraformaldehyde for 48 h, and stored in 70% ethanol until scanned on a micro-CT imaging system (Skyscan 1076; Bruker, Kontich, Belgium). All micro-CT scanning parameters and measurements were performed as described previously^{51–53}. Briefly, maxillae were scanned using 9 µm (0.01 mm) isotropic spatial resolution, 0.5 mm aluminum filter, 55 kV voltage, 181 µA current, 195 ms exposure, frame averaging of 10, and rotation step 0.4° across 180°. For reconstruction (NRecon 1.6.1.5; Bruker), the following parameters were used: ring

artifact correction, 5; pixel defect mask, 5%; smoothing, 1%; beam hardening correction, 20%, and compressed sensing (CS) to image conversion, 0.0–0.8.

All the images were oriented and saved in axial slices (2000×1336) using specific software (Data Viewer 1.6.1.5; SkyScan, Kontich, Belgium). The linear measurements were determined using the Data Viewer software according to the defined anatomical landmarks measuring the distance from the cement-enamel junction (CEJ) to the alveolar bone crest (ABC) in the distal region of the first molar buccal root and the mesial region of the second molar buccal root. To standardize the micro-CT sections, the long axis of the tooth was parallel to the sagittal and coronal axes and perpendicular to the axial axis. The CEJ of the second molar was parallel in the sagittal plane and coronal planes. The two linear sample measurements resulted in an average value, which was used to define the distance from the CEJ to the ABC in millimeters.

The volumetric analysis was performed using a specific program (CTAnalyzer 1.10.1.0; SkyScan, Kontich, Belgium) and a region of interest (ROI) was defined in an axial orientation from the root apices to the alveolar crest, and from the mesial root of the first molar to the mesial root of the second molar. For all teeth, volumetric bone levels were assessed in the axial plane. The volumetric measurements started 10 slices below the CEJ going towards the teeth apices, representing the bone level distances. Volumetric measurements were taken until the root apices. This ROI was manually delimited every ten planes extending 80 slices. The roots of the teeth and periodontal ligaments were excluded from the ROI. The microarchitecture parameters evaluated were: bone volume (BV), tissue volume (TV), and bone volume fraction (BV/TV).

Bone mineral density (BMD) was also measured. The calibration for BMD measurements was performed with the two calcium hydroxyapatite (CaHA) phantom rods containing two different concentrations of 0.25 and 0.75 g of CaHA/cm³. The

attenuation coefficient was entered into the calibration dialogue window in CTAn to determine BMD (mg/cm³).

4.2.2.6 Histological stereometric analysis

The analysis was performed using an optical microscope coupled to a color digital camera using standardized settings for image acquisition (Leica Application Suite 3.8, Wetzlar, Germany). The sections were processed for regular hematoxylin and eosin (H&E) staining. Six semi-serial sections of 5- μ m thickness spaced 100 μ m from each other (spanning 630 μ m of the buccolingual aspect of the first and second maxillary molars) were obtained from each one of the tissue blocks from six different animals in each experimental condition. A 17,765- μ m² grid with 10x5 squares of 30 μ m was constructed using an image-editing software (Adobe Photoshop CC, Adobe System Incorporated, San Jose, CA, USA). It was overlaid on the digital histological images obtained at $\times$ 200 magnification, allowing the analysis of a defined region of interest (ROI). The whole grid representing the ROI for the analysis was represented by the whole grid, which was positioned in a supracrestal area, representing the connective tissue subjacent to the gingival sulcus and above the bone crest (the interproximal region between the first and second maxillary molars).

The analysis was performed using a point-counting technique, as described⁵³⁻⁵⁶. This point-counting technique was used to evaluate the proportion of the following structures: fibroblasts cells, inflammatory cells, vascular structures, and extracellular matrix on the H&E-stained sections. The type of structure found at the intersection of the grid lines was counted on an optical microscope. The results were expressed as a percentage of the total area analyzed.

4.2.2.7 TRAP staining

The presence of osteoclasts in the histological sections was also verified by biochemical staining of tartrate-resistant acid phosphatase (TRAP) (Leukocyte Acid Phosphatase 387-A, Sigma-Aldrich, St. Louis, MO, USA). Sections were deparaffinized, rehydrated, fixed in a fixative solution for 30 seconds, and then incubated at 37°C in the TRAP staining solution for 60 minutes, as described³⁻⁵³. After counterstaining with Harrys' hematoxylin, sections were dried and cover slipped. Digital images were obtained at 40x magnification using standardized acquisition parameters. The total numbers of TRAP-positive multinucleated cells containing 3 or more nuclei were counted in the furcation area of the first maxillary molar (from the most coronary point to the root apex). The area considered in this analysis was calculated using the image analysis software (Image-Pro Express 6.0, Olympus, Japan). The number of TRAP-positive multinucleated cells in the bone area was determined in the region-of-interest (furcation area) analyzed.

4.2.2.8 Immunohistochemistry

IHC analysis was performed to evaluate the protein expression of CD45- (a marker of leukocyte), CD3- (a marker of T cells), MAC387- (a marker of macrophages), and IL-1 β ; within the periodontal tissues in the interproximal region of the first maxillary molars. Serial parasagittal sections with 5- μ m thickness were mounted on silanized slides. The analysis was accomplished according to a previously published study⁵⁵.

IHC technique was performed by the avidin-biotin peroxidase (ABC) method using the LSAB kit (Dako, Glostrup, Denmark) according to manufacturer's instructions. Tissue sections were deparaffinized, rehydrated through a graded ethanol series, and then washed with phosphate buffer solution (PBS). Antigen retrieval was carried out by hitting cuts in the Rodent Decloaker reagent (Biocare Medical; Concord, CA) in a Decloaking

Chamber (Biocare Medical) for 30 min at 80°C. Endogenous peroxidase activity was blocked with a 5% hydrogen peroxide solution and nonspecific binding blocking was performed in 5% bovine serum albumin (BSA, Sigma) in PBS. As a negative control, primary antibody was omitted and the sections were incubated with 1% PBS to assess background staining.

Sample sections were incubated overnight with a primary antibody against the following proteins antibody (CD3 1:200, ab16669; MAC387 1:200, ab22506; CD45 1:600, ab64100; IL-1 β 1:450, ab9722; Abcam, Cambridge, UK). The samples were then washed extensively, and further incubated with appropriate secondary antibodies for 1 h; slides were stained with DAB (Dako) and counterstained with Carazzi's hematoxylin.

The samples were visualized using an Olympus camera (DP-71, Olympus, Tokyo, Japan) attached to a light microscope (BX51, Olympus, Tokyo, Japan) at 40x magnification for CD3, CD45, and MAC387, and at 100x magnification for IL-1B. Positive cells present in the region were counted by a blinded and calibrated examiner. The determination of the intensity and area of chromogen staining were performed as quantitative analysis, according to a previous study⁵⁷.

4.2.2.9 Reverse transcriptase and quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from tissue samples using the RNeasy kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. The RNA concentration was determined by spectrophotometry, and 700 ng of total RNA was reversely transcribed into cDNA using random hexamers primers (High Capacity cDNA synthesis kit). The relative abundance of the transcripts of the candidate inflammatory gene was measured by RT-qPCR using Taqman chemistry and predesigned sets of primers and probes (TaqMan Gene Expression Assays; Applied Biosystems) on a

StepOne Plus Real-Time PCR System (Applied Biosystems). The reactions were carried out in a 96-well plate in a final reaction volume of 30 μ L including Taqman Universal PCR Master Mix (Applied Biosystems), cDNA template (corresponding to 30 ng of cDNA, and Taqman Gene Expression Assays (Applied Biosystems) for each target gene: TNF- α , Mm00443259_g1; and IL-1 β (interleukin 1 β), Mm01336189_m1. Optimized thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. For each sample, analyses of gene expression were performed in duplicate. To normalize the amount of mRNA present in each reaction, the expression of GAPDH was used as a housekeeping gene.

4.2.3 In vitro experiments

4.2.3.1 Osteoclastogenesis assay

For all in vitro experiments, bone marrow macrophages (BMM) from male mice aging between 4 and 6 weeks were isolated from the femur and tibia and resuspended in ice-cold water to remove red blood cells. After centrifugation, cells were cultured in α -Minimal Essential Medium supplemented with 10% fetal calf serum and penicillin (100 μ g/mL) and streptomycin (100 μ g/mL) containing 30 ng/mL M-CSF in a suspension culture dish, to which stromal and lymphoid cells can not adhere. Two days later, the adhering BMMs were detached and seeded in 96 wells flat bottom tissue culture plates at a density of 5×10^3 cells per well in 100 μ L culture medium or in 6 well plates at a density of 1×10^5 in 2 mL culture medium (for Western blot) containing either 30 ng/mL recombinant murine M-CSF, M-CSF + 4 ng/mL recombinant murine RANKL or M-CSF + RANKL + CsinCPI-2 (100 μ M unless stated otherwise). Half of the media containing all the substances for each group was replaced on day 3. At the end of the culture periods (1, 2, 3 and 4 days), wells were washed with PBS, and the cells were either fixed in 4%

PBS-buffered paraformaldehyde and stored at 4 °C (used for TRAP staining and actin ring formation), dissolved in RNA lysis buffer from RNAqueous®-Micro Kit and stored at -80 °C for RNA isolation or were lysed with Mammalian Protein Extraction Reagent (M-PER).

4.2.3.2 Tartrate-resistant acid phosphatase (TRAP) staining

Fixed cells on plastic were stained for TRAP using the leukocyte acid phosphatase kit according to the manufacturer's instructions. TRAP-positive cells with 3 or more nuclei were considered osteoclasts, and the number of multinucleated osteoclasts was counted (TRAP+ MuOCL) manually in an inverted microscope.

4.2.3.3 Fluorescence microscopy - actin ring formation of osteoclasts

To examine the formation of actin rings, cells were washed with PBS and fixed with 4% buffered formaldehyde in PBS for 2 minutes at room temperature. Fixed cells were washed three times with PBS, permeabilized with 0.2% Triton X, and 1% BSA-PBS for 20 min. Subsequently, the cells were incubated with the rhodamine-conjugated phalloidin (Thermo Fisher Scientific) (1:40) for 30 minutes at room temperature, as previously described ¹⁶. Actin rings were visualized fluorescently using a Leica DMI 6000B microscope (Leica Microsystems, Inc, Richmond Hill, ON).

4.2.3.4 RNA isolation and first-strand cDNA synthesis

Total RNA was extracted using the commercially available RNAqueous®-Micro total RNA extraction kit by following the manufacturer's protocol. RNA was reversed and transcribed into single stranded cDNA with a commercially available cDNA synthesis kit using High-Capacity cDNA Reverse Transcription Kit.

4.2.3.5 Quantitative real-time polymerase chain reaction (RT-qPCR)

Quantitative real-time PCR analysis was performed using SyBr Green Universal PCR master Mix. In each reaction, cDNA was amplified using aPowerUp™ SYBR™ Green Master Mix, 300 nmol/L of each primer and on a StepOnePlus Real-Time PCR system.

The primers used were *Ctsk* (Forward: 5'-ATATGTGGGCCAGGATGAAAGTT-3'; Reverse: 5'-TCGTTCCCCACAGGAATCTCT-3'), *Acp5* (Forward: 5'-CGACCATTGTTAGCCACATACG-3'; Reverse: 5'-TCGTCCTGAAGATACTGCAGGTT-3'), *Calcr* (Forward: 5'-AGTTGCCCTCTTATGAAGGAGAAG-3'; Reverse: 5'-GGAGTGTCGTCCCAGCACAT-3'), *Nfatc1* (Forward: 5'-TGAGGCTGGTCTTCCGAGTT-3'; Reverse: 5'-CGCTGGGAACACTCGATAGG-3'), *Pbgd* (Forward: 5'-AGTGATGAAAGATGGGCAACT-3'; Reverse: 5'-AGTGATGAAAGATGGGCAACT-3') was used as internal standard to correct for differences in starting mRNA concentrations.

4.2.3.6 Western blot analysis

For Western blot analysis, cells from 3 wells of a 6-well plate were lysed with 100 µL of Mammalian Protein Extraction Reagent (M-PER). Total protein was quantified in each sample using a BCA assay kit, and protein samples (20µg) were then separated by SDS-PAGE and transferred onto nitrocellulose membranes. For detection, the membranes were incubated with Nfatc1 primary antibody (1:500) overnight at 4°C in 1% BSA/PBS. Three times 10 min of wash in TBS with 0.05% Tween-20 (TBST) was followed by incubation with HRP-conjugated secondary antibody (1:5,000 in 1% BSA, 0.05% Tween-20 in TBS) for 60 min at room temperature. Finally, membranes were

washed extensively with TBST and TBS, followed by development using a chemiluminescence detection kit (ECL Plus Western Blotting Substrate) according to the manufacturer's protocol.

4.2.3.7 MTT assay

To determine cellular viability in the different groups, a time course Methyl tetrazolium (MTT) (Sigma-Aldrich) assay was performed after 1, 2, 3, and 4 days of culture. The culture media at the end of each time point was replaced by 90 μ L of α -MEM (Gibco) and 10 μ L of a 5 mg/mL MTT solution in PBS. After 4 hours of incubation at 37 $^{\circ}$ C, 100 μ L of acid-isopropanol solution (0.04 N HCl in isopropanol) was added to each well to dissolve the formazan crystals formed and incubated for 15 min under gentle shaking (VDRL Shaker, Biomixer). Absorbance was read in a microplate reader at 570 nm (Gen 5, Epoch, BioTek Instruments).

4.3 Statistical Analysis

All statistical analyses were conducted using GraphPad Prism (GraphPad Prism 7.0, San Diego, CA, USA). In vitro data is representative from 2-3 experiments. The normality of data distribution was verified with the Shapiro-Wilk test. Depending on the data distribution for a particular outcome, two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test for multiple comparisons among groups and Kruskal-Wallis followed by the Dunn post hoc test were used. The significance level was set at 5% ($P < 0.05$). Parametric data is expressed as the mean $\pm$ SD, and nonparametric data is expressed as median and range.

5 RESULTS

5.1 In Vivo Experiment

5.1.1 Systemic administration of CsinCPI-2 protein did not induce adverse effects in mice

All animals were accompanied throughout the entire experimental period to evaluate any signs of adverse effect that the systemic administration of *CsinCPI-2* would result in. None of the mice developed any signs or symptoms of adverse effects caused by daily administration of the compound. No animals were lost during the experimental period.

5.1.2 CsinCPI-2 diminishes alveolar bone loss by decreasing TRAP+ osteoclast in vivo

To investigate the effects of daily *CsinCPI-2* administration on bone resorption, mice underwent ligature placement to induce PD and were treated for 15 days with *CsinCPI-2* protein until sacrifice. Maxillary bone in the CTL group was characterized by a normal alveolar crest height located near the CEJ (cementoenamel junction) and completely filling the furcation area, indicating no sign of bone loss (Figure 1A). Ligated maxillary molars (PD group) had marked alterations in bone morphology with advanced reduction of bone height in the interproximal and furcation areas (Figure 1A). *CsinCPI-2* treatment markedly reduced bone destruction, pointing to an important role of this compound as an inhibitor of inflammation-induced bone loss (Figure 1A).

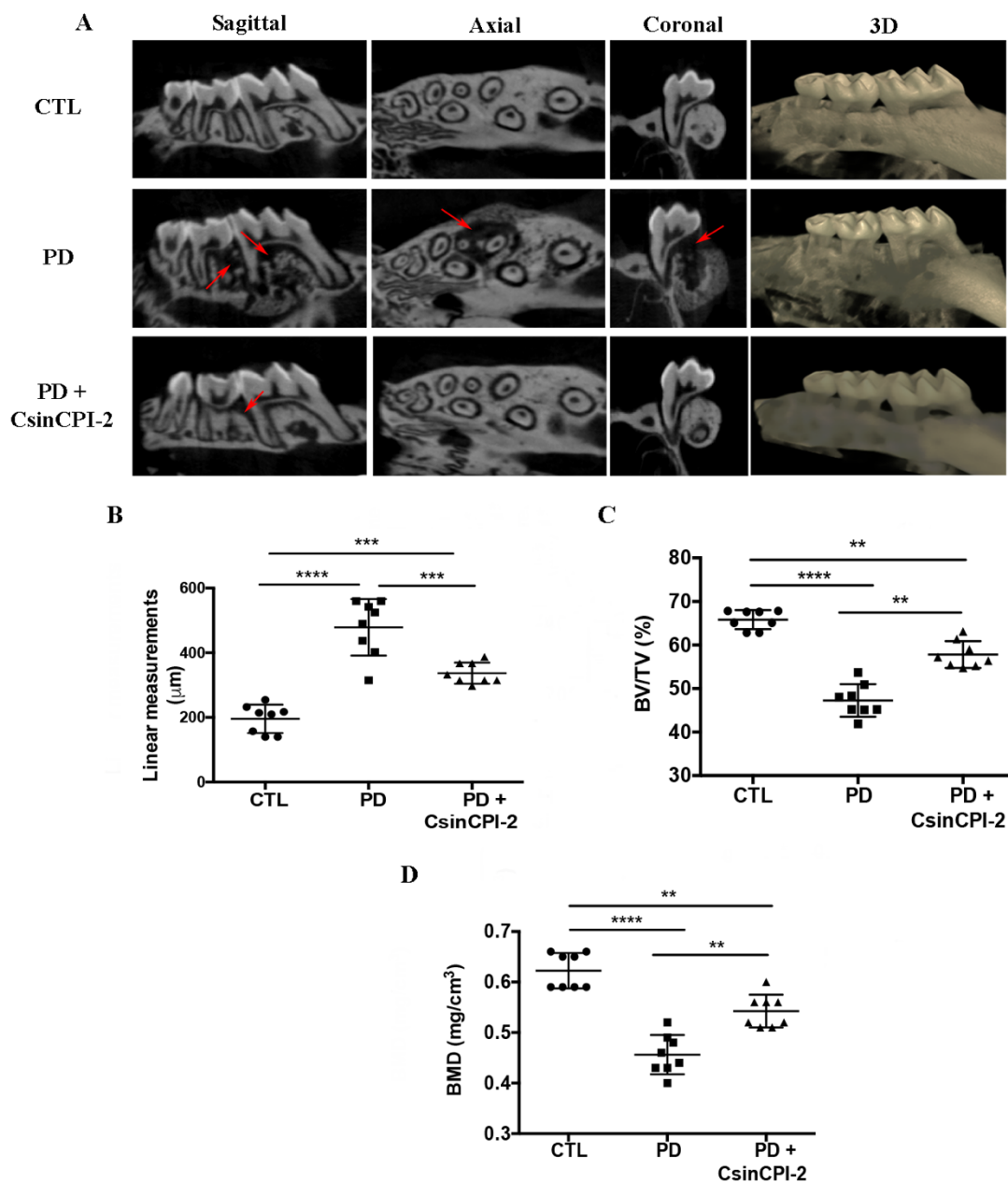
Quantitative analysis did show significant differences in alveolar crest height among groups, as evidenced by linear measurements (Figure 1B) Reflecting the largest amount of bone loss observed in the experimental groups, the PD group exhibited a significantly increased distance between CEJ and ABC (alveolar bone crest) compared to the CTL group, which was significantly reduced by treatment with *CsinCPI-2*.

Volumetric measurements and evaluation of bone mineral density were also

evaluated. Our findings demonstrated that *CsinCPI-2* significantly inhibited bone loss without significant difference with the CTL group. The PD group as expected, presented with the lowest values of BV/TV (Figure 1C) and was significantly different compared to the CTL and PD+*CsinCPI-2* groups. Notably, *CsinCPI-2* treatment also increased BMD compared to the PD group (Figure 1D).

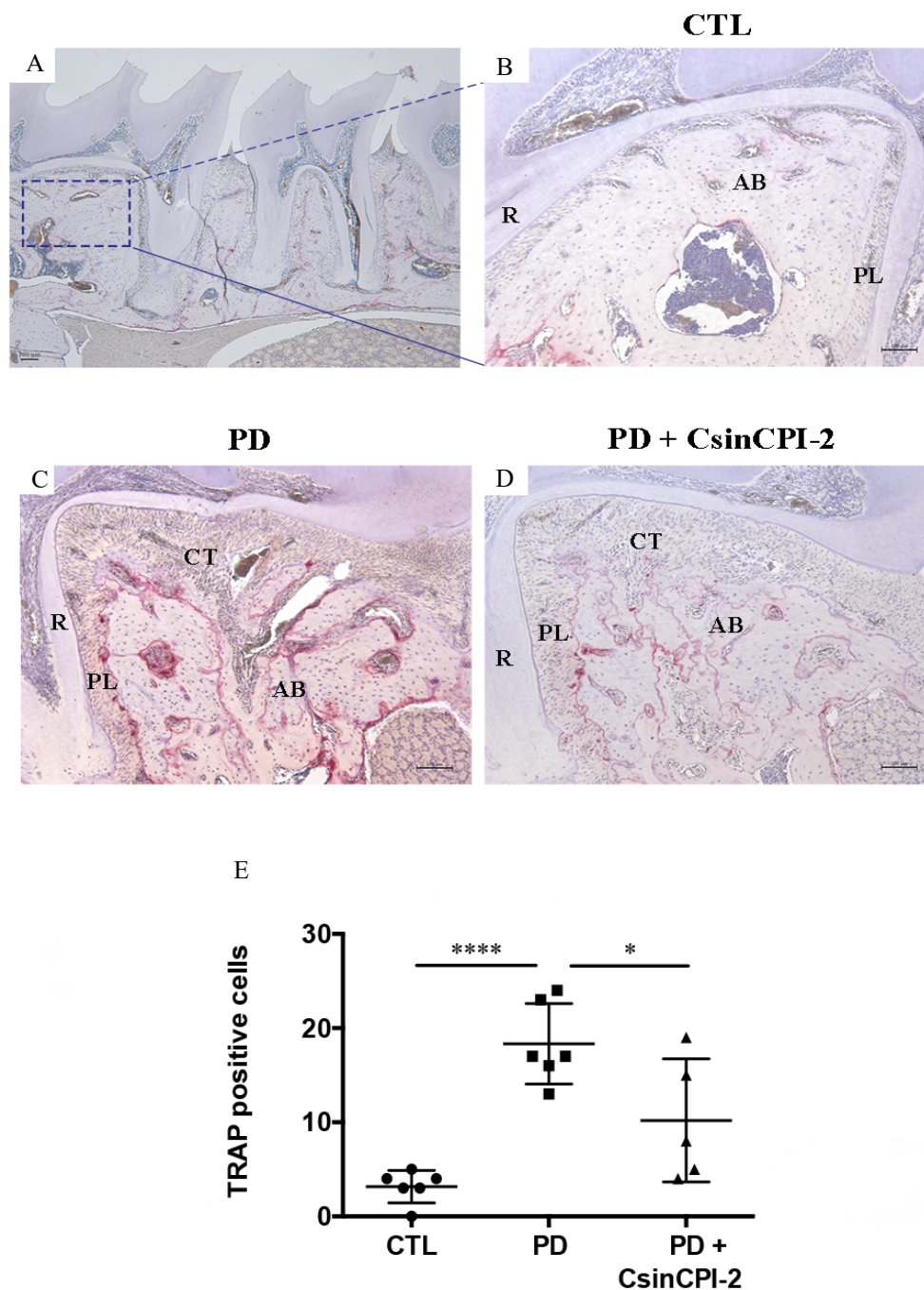
To examine the effect of *CsinCPI-2* on osteoclastogenesis, maxillary tissue sections were stained for TRAP activities. The results indicated that the CTL group presented with few TRAP⁺ osteoclasts in the furcation area between first and second molars (Figure 2 A-B). On the other hand, the PD group showed significant increase in the number of TRAP⁺ osteoclasts (Figure 2C). Remarkably, *CsinCPI-2* treatment significantly diminished the osteoclast number compared to the PD group (Figure 2D). Quantitative analysis of TRAP⁺ osteoclasts demonstrated a significant increase in osteoclast numbers in the PD group compared to the CTL group, and that *CsinCPI-2* significantly decreased the enhanced numbers in the PD group (Figure 2E).

Figure 1 - A) Three-dimensional reconstruction (sagittal, axial, coronal, and 3D reconstruction views) of the alveolar bone in all groups (A). Data from (B) linear measurements (distance between the cement-enamel junction and the alveolar bone crest); (C) BV/TV – percentage of bone volume in the ROI (region of interest – around 1st molar roots) and (D) bone mineral density (BMD) (n=8). Data represents the mean $\pm$ SD. Ordinary one-way ANOVA followed by Tukey post hoc test * $P<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.



Source: Self elaboration.

Figure 2 - Representative image of the area of interest (furcation area in the first maxillary molar) in the histological sections stained for TRAP (A). Representative images from groups CTL, PD, and PD + CsinCPI-2, respectively (B-D). (E) Quantification of the numbers of TRAP+MuOCL. Data represent the mean $\pm$ SD. Ordinary one-way ANOVA followed by Tukey post hoc test * $P < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$



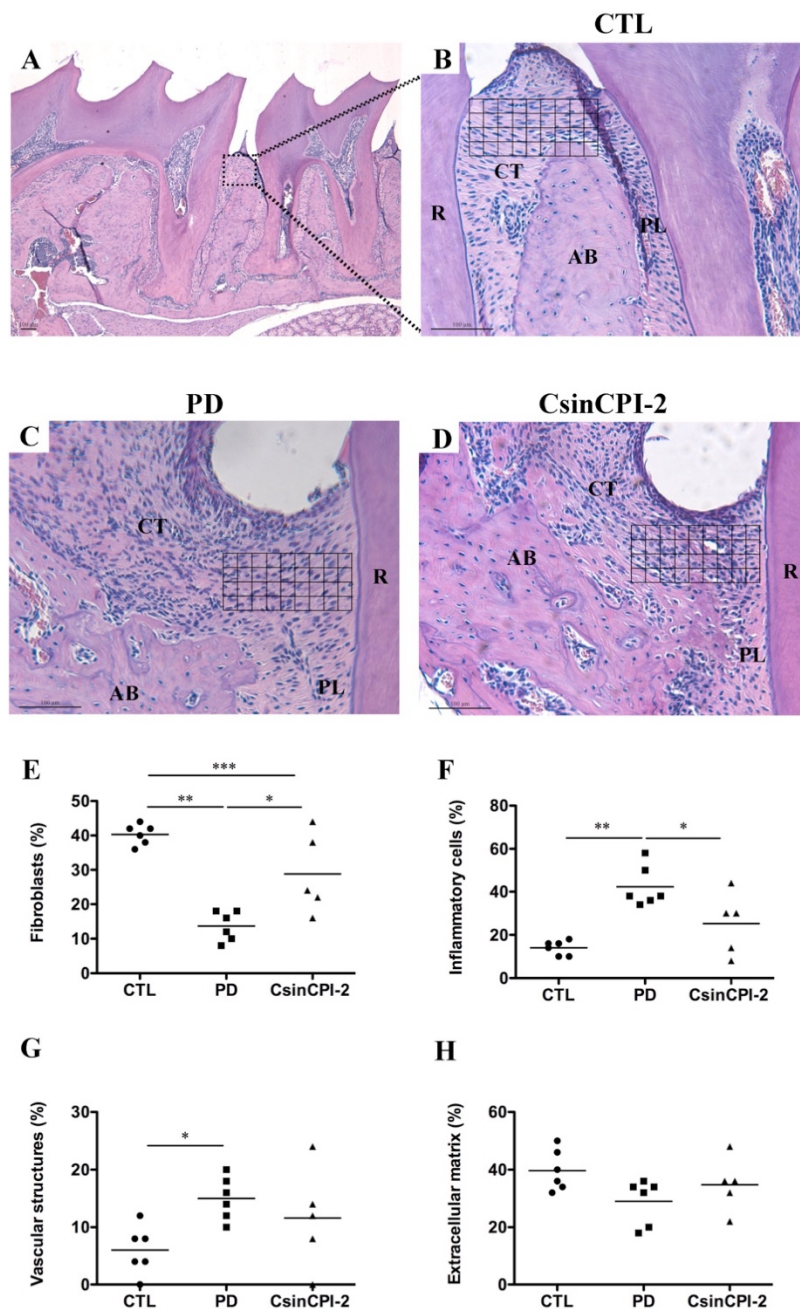
Source: Self elaboration.

5.1.3 Systemic treatment with CsinCPI-2 protein attenuated inflammatory infiltrate in periodontal tissues

Based on the decreased bone resorption and the reduced number of TRAP⁺ osteoclasts, we then explored the results of daily administration of *CsinCPI-2* on the severity of inflammation, in a specific area in the connective tissue corresponding to the site where the ligature was placed. Analysis of H&E-stained sections indicated that the CTL group presented with few inflammatory cells in the connective tissue, normal alveolar bone crest in the interproximal region and absence of inflammatory infiltrate (Figure 3A-B). Ligatures in the PD group led to increased inflammatory infiltrate in the connective tissue just below the area where ligature was placed, with loss of bone mass with epithelial downgrowth (Figure 3C). Notably, histological sections in animals treated with *CsinCPI-2* did show diminished inflammatory infiltrate and epithelial downgrowth compared to the PD group (Figure 3D).

Stereometric analysis indicated that administration of *CsinCPI-2* significantly increased the proportion of fibroblasts compared to the PD group but did not reach the levels of the CTL group (Figure 3E). Notably, the increased percentage of inflammatory cells was significantly different when animals were treated with *CsinCPI-2* compared to the PD group, which indicates the anti-inflammatory effect of *CsinCPI-2*. No differences were observed between CTL and PD+*CsinCPI-2* groups on the proportion of inflammatory cells (Figure 3F). Vascular structures (Figure 3G) and extracellular matrix were not significantly different between PD+*CsinCPI-2* and PD groups.

Figure 3 - Daily administration of CsinCPI-2 significantly reduced inflammation and increased fibroblast proportion in the interproximal region of the upper jaw. (A) H&E-stained slides are showing the ROI where the analyses were performed. (B-D) H&E-stained sections in CTL, PD, and PD + CsinCPI-2 groups, respectively. A grid was overlaid on the ROI, and percentage analysis of the tissue components was performed in relation to the total number of the grid intersections counted. (E-H) Quantification of stereometric findings, i.e., percentage of the grid intersections over fibroblasts, inflammatory cells, vascular structures, and extracellular matrix, respectively. Graphs represent quantitative analysis from 8 animals in each experimental condition. Data represents the mean $\pm$ SD. Analysis was performed by Ordinary one-way ANOVA followed by the Tukey post hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.



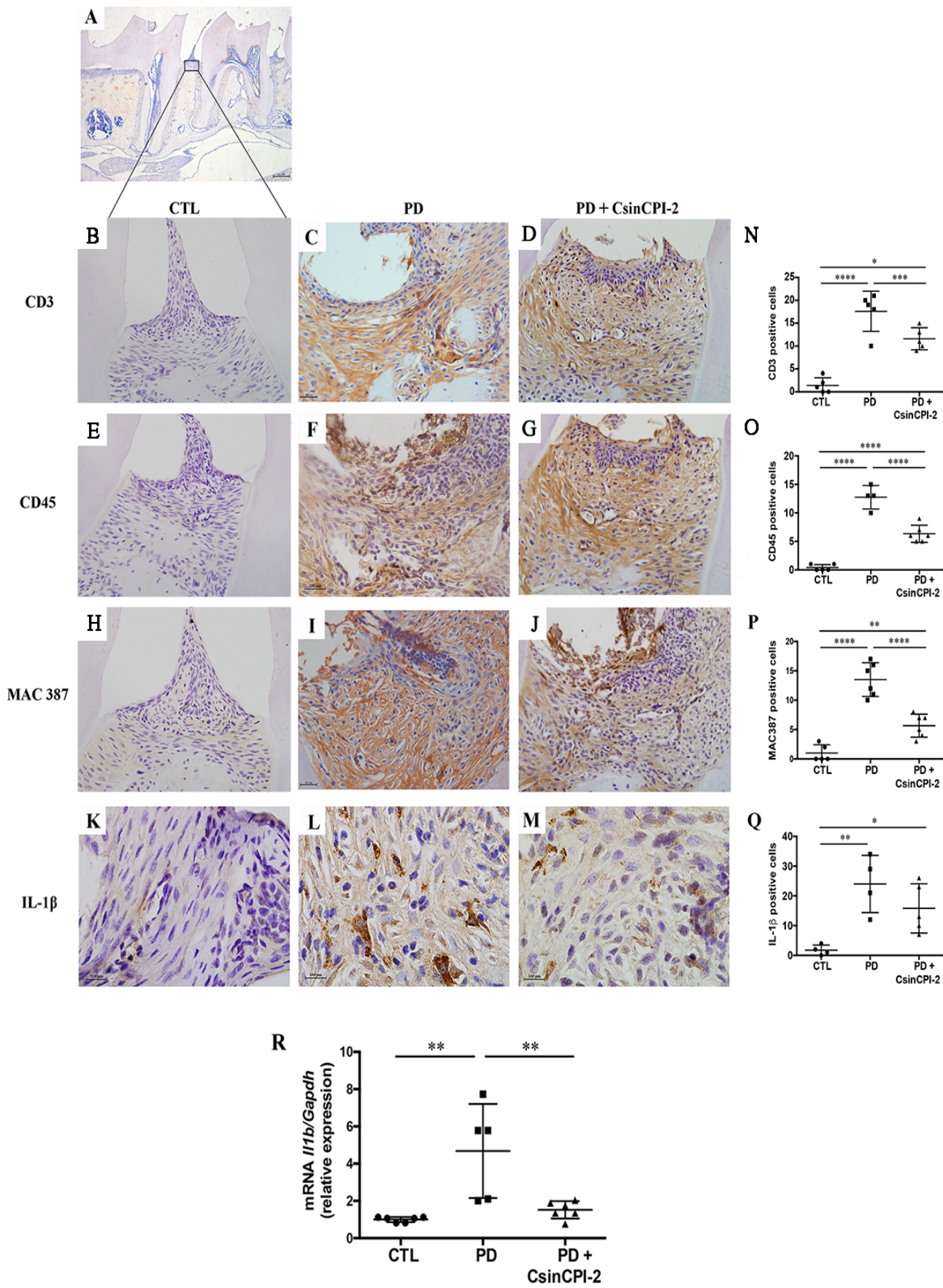
Source: Self elaboration.

5.1.4 CsinCPI-2 protein down regulated protein levels of inflammatory markers CD3, CD45, MAC387, and IL-1 β

To further strengthen the inflammation-based findings upon the stereometric analyses, three different markers of inflammatory cells were investigated by IHC (Figure 4A). CD3, a marker of T lymphocytes (Figure 4B-D); CD45, a marker of leukocyte (Figure 4E-G); and MAC387, a marker of macrophages (Figure 4H-J), were stained in the histological sections. Moreover, inflammatory cytokine IL-1 β was also analyzed (Figure 4K-M). Our data demonstrated that the CTL group presented with the lowest percentages of staining for all the markers evaluated, which were significantly different compared to the PD and PD+CsinCPI-2 groups (Figure 4N-Q). Importantly, CsinCPI-2 significantly decreased protein expression of CD3+, CD45+ and MAC387+ cells when compared to the PD group according to the figures 4A-Q.

In agreement with these IHC observations, the mRNA expression of *Il1b* in the gingival tissues around the first maxillary molar in intimate contact with the ligatures was significantly increased in the PD group; a response downregulated by CsinCPI-2 (Figure 4R).

Figure 4 - CsinCPI-2 down-regulated protein expression of candidate markers of inflammation: CD3, CD45, MAC387, and IL-1 β in the region of interest (ROI) analyzed. (A) Representative IHC-stained slides of the ROI and the number of cells positively stained with antibodies to CD3 (B-D), CD45 (E-G), MAC 387 (H-J), and IL-1 β (K-M) of CTL, PD, and PD + CsinCPI-2 groups, respectively. Quantification of staining percentage of CD3 (N), CD45 (O), MAC387 (P), and IL-1 β (Q). (R) Ligature increased the mRNA expression of *Il1b* in the PD group, and CsinCPI-2 significantly reduced this enhancement. mRNA levels were assessed by qPCR and normalized to those of GAPDH, and the values shown are expressed as fold change to the control group. Graphs represent quantitative analysis from 6 animals in each experimental condition (mean $\pm$ SD). Statistical analysis was assessed by one-way ANOVA followed by Tuckey. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.



Source: Self elaboration.

5.2 In Vitro Experiment

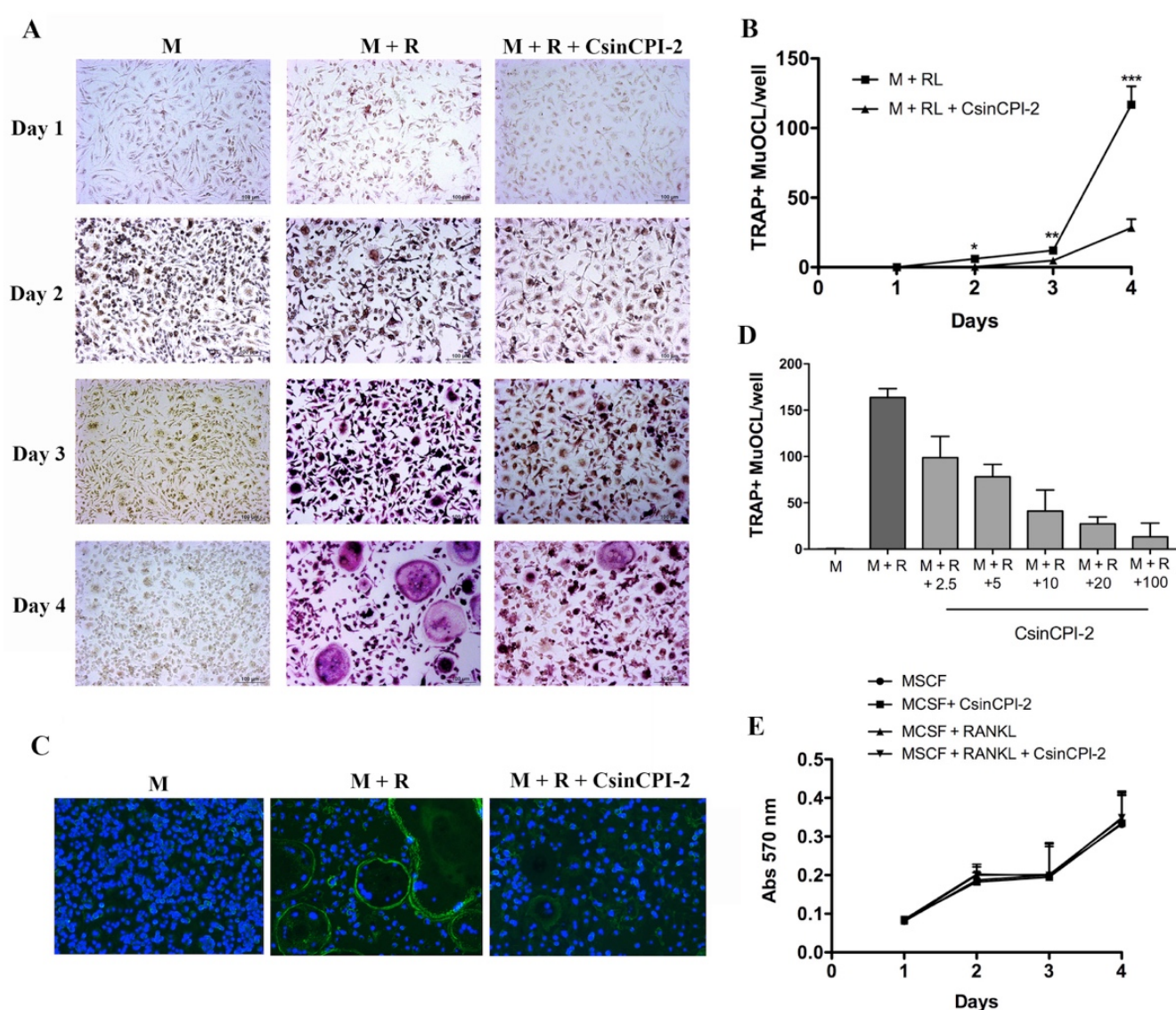
5.2.1 *CsinCPI-2* inhibits osteoclastogenesis by decreasing osteoclasts differentiation

To investigate if the decreased numbers of osteoclasts observed in the PD group treated with *CsinCPI-2* might be due to a direct effect of *CsinCPI-2* on osteoclast differentiation, mouse BMMs were treated with M-CSF (30 ng/ml), and RANKL (4 ng/ml), in the absence or presence of *CsinCPI-2* (100 μ M). After 1-4 days of treatment the numbers of TRAP+MuOCLs were counted. Figure 5A demonstrates that addition of *CsinCPI-2* significantly inhibited the formation of mature TRAP+MuOCL induced by M-CSF/RANKL. The inhibitory effect of *CsinCPI-2* was significant at day 2 and very robust at day 5 (Figure 5B).

Next, the effect of *CsinCPI-2* on osteoclast function was investigated. Cells were incubated with different concentrations of *CsinCPI-2* (2.5, 5, 10, 20, 100 μ M) and actin ring formation on osteoclasts was evaluated to demonstrate that the TRAP+MuOCL formed had signs of mature osteoclasts. *CsinCPI-2* significantly reduced the formation of cells with actin rings in a concentration-dependent manner (Figure 5C). The inhibitory effect was apparent at 2.5 μ M and gradually increased with enhanced concentrations of *CsinCPI-2* with half-maximal effect at 5 μ M (Figure 5D).

To further investigate if the inhibitory effect of *CsinCPI-2* was not due to cytotoxicity, we performed the MTT assay in BMMs and osteoclasts cultured for 1 to 4 days (Figure 5E). As shown in Figure 5E, the *CsinCPI-2* (100 μ M) compound did not affect MTT activity in BMMs (M-CSF) or in osteoclasts (M-CSF + RANKL), which indicate that inhibition of osteoclast formation was not due to cellular toxicity.

Figure 5 - Mouse BMMs were treated with either M-CSF (M; 30 ng/ml), M-CSF and RANKL (M+R; 4 ng/ml), or M-CSF, RANKL and CsinCPI-2 (M + R + CsinCPI-2 40 μ M). After 2 and 4 days of treatment, the cells were lysed for RT-qPCR. CsinCPI-2 prevents the up-regulation of *Calcr*, *Acp5*, and *Ctsk* mRNA induced by RANKL (A-F). The relative expression of *Nfatc1* mRNA induced by RANKL (G, H) was significantly abrogated when cells were additionally treated with CsinCPI-2, an effect which slightly decreased after 4 days. Finally, western blot for *Nfatc1* protein expression demonstrated complete inhibition by CsinCPI-2 of RANKL-stimulated *Nfatc1* at day 2 and partial inhibition at day 4 (I). Values for (A-L) represent means $\pm$ SD (n=6). Ordinary one-way ANOVA followed by Tukey post-test. *p < 0.05, ** p < 0.01; *** p < 0.001; **** p < 0.0001.



Source: Self elaboration.

5.2.2 Osteoclastogenesis inhibited by CsinCPI-2 is due to retention of the bone marrow macrophages at the macrophage stage.

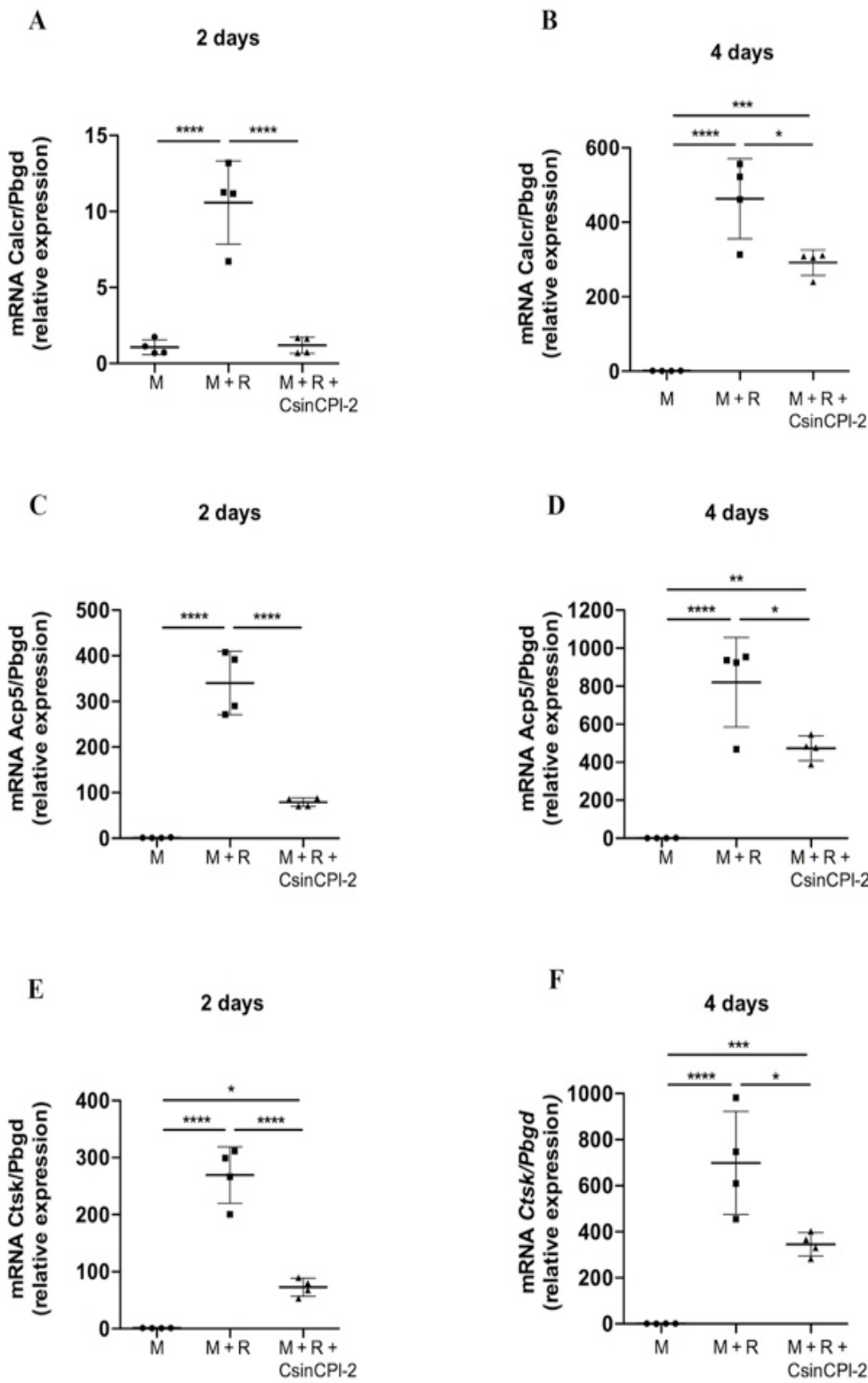
To further evaluate the inhibition of osteoclastogenesis by *CsinCPI-2* treatment, mouse BMM were treated with M-CSF (30 ng/ml), and RANKL (4 ng/ml), in the presence of *CsinCPI-2* (100 μ M), and mRNA expression of osteoclast phenotypic markers were investigated. Our findings demonstrated that *CsinCPI-2* was able to significantly inhibit RANKL-induced mRNA expression of the osteoclast phenotypic markers *Calcr* (encoding calcitonin receptor), *Acp5* (encoding tartrate-resistant acid phosphatase), and *Ctsk* (encoding cathepsin K) (Figure 6A-F) on days 2 and 4, showing that the decreased numbers of multinucleated osteoclasts are due to interference by *CsinCPI-2* on differentiation of mononucleated osteoclast progenitors rather than inhibition of their fusion.

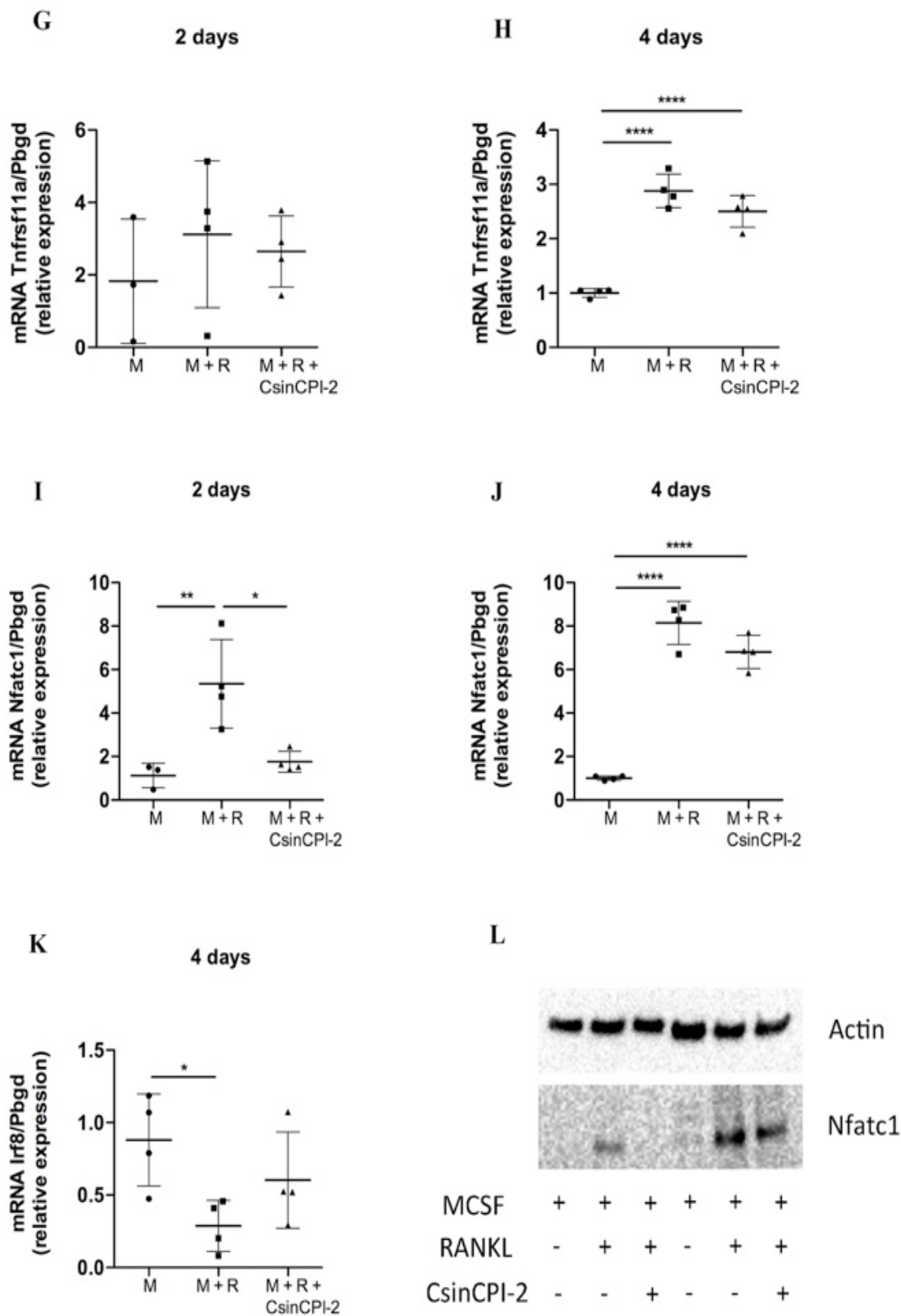
5.2.3 Osteoclastogenesis inhibition by CsinCPI-2 is due to inhibition of the expression of *Nfatc1*.

To assess the mechanisms by which *CsinCPI-2* inhibits osteoclastogenesis, the expression of genes crucial to osteoclast differentiation, receptor activator of nuclear factor- κ B (*Rank*) and nuclear factor of activated T-cells, cytoplasmic 1 (*Nfatc1*), and macrophage marker *Irf8* were investigated by quantitative real time polymerase chain reaction (RTq-PCR) at 2 and 4 days after initiation of experiments. The real-time q-PCR analyses revealed that the addition of *CsinCPI-2* (100 μ M) did not decrease the mRNA expression of *Tnfrsf11a* (encoding RANK) in BMMs stimulated by M-CSF and RANKL for 2 and 4 days (Figure 6 G, H), suggesting that the mechanism of action by which *CsinCPI-2* inhibits osteoclastogenesis is down-streamed to RANK. In agreement with this suggestion, RANKL-induced increased mRNA expression of *Nfatc1* was significantly abrogated when *CsinCPI-2* was added to the culture media both after 2 and 4 days (Figure 6 I, J). Down-regulation of the mRNA expression for the macrophage

marker *Irf8* by RANKL was partially prevented by *CsinCPI-2*, 4 days after cell stimulation (Figure 6 K). Importantly, the RANKL-induced up-regulation of NFATC1 protein was also inhibited by *CsinCPI-2* at 2 and 4 days (Figure 6 L).

Figure 6 - Mouse BMMs were treated with either M-CSF (M; 30 ng/ml), M-CSF and RANKL (M+R; 4 ng/ml), or M-CSF, RANKL and *CsinCPI-2* (M + R + *CsinCPI-2* 40 μ M). After 2 and 4 days of treatment, the cells were lysed for RT-qPCR. *CsinCPI-2* preventing the up-regulation of *Calcr*, *Acp5*, and *Ctsk* mRNA induced by RANKL (A-F). The relative expression of *Nfatc1* mRNA induced by RANKL (G, H) was significantly abrogated when cells were additionally treated with *CsinCPI-2*, an effect which slightly decreased after 4 days. Finally, western blot for *Nfatc1* protein expression demonstrated complete inhibition by *CsinCPI-2* of RANKL-stimulated *Nfatc1* at day 2 and partial inhibition at day 4 (I). Values for (A-L) represent means $\pm$ SD (n=6). Ordinary one-way ANOVA followed by Tukey post-test. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$





Source: Self elaboration.

6 DISCUSSION

The present study demonstrated that the new phytocystatin *CsinCPI-2* can inhibit osteoclastogenesis in vitro and in vivo, suggesting a potential therapeutic application of this protein mainly for inflammatory disease-associated bone loss.

The in vivo results showed that phytocystatin *CsinCPI-2* prevented periodontal destruction in an experimental periodontitis mice model, demonstrated by reduced alveolar bone loss, which was associated with reduced number of TRAP⁺ osteoclasts and inflammatory cells, observed by immunohistochemistry for leukocyte (CD45) and more specifically for macrophages (MAC387) and T-cells (CD3). In addition, decreased gene expression and protein level of pro-inflammatory cytokine IL-1 β were observed in the periodontal tissues. Importantly, no adverse effects were associated with the systemic administration of *CsinCPI-2*. Together, this data confirms the anti-inflammatory effect of *CsinCPI-2* in the periodontitis model and corroborates our previous study, which showed that *CsinCPI-2* inhibited cysteine peptidases (CtsK and CtsB) and pro-inflammatory cytokines (TNF- α and IL-1 β) in vitro in RAW 264.7, a mouse macrophage cell line¹⁰.

In Periodontitis and other inflammatory diseases, as rheumatoid arthritis, bone loss is characterized by an imbalance on bone resorption and apposition in the bone surface adjacent to an inflamed connective tissue²⁵⁻³⁶. Although a complex mechanism is involved, enhanced osteoclastogenesis is mainly promoted by an increased expression of RANKL by inflammatory cells or by resident cells, as osteoblasts and fibroblasts, stimulated by pro-inflammatory cytokines produced in the inflamed tissue²⁵⁻²⁷⁻³⁶⁻⁵⁸. Among these cytokines, IL-1 β is a pivotal molecule in osteoclast activation acting directly and indirectly in the process of osteoclastogenesis⁵⁹⁻⁶⁰. It has been reported that IL-1 inhibition is associated with decreased bone loss²⁵⁻⁶¹. Studies demonstrated that loss of connective tissue attachment and alveolar bone in PD can be retarded using specific

antagonists of IL-1 and TNF²⁸. IL-1 induces TNF- α and works with it to promote bone resorption⁶¹. These cytokines help leukocytes infiltrate the surrounding tissue and stimulate secondary mediators, leading to an amplification of the inflammatory response²⁶. In this study, leukocyte concentration and IL-1 β expression were reduced in the supra-alveolar connective gingival tissue when *CsinCPI-2* was administered concomitantly with experimental periodontitis induction.

Therefore, our results suggest that the anti-inflammatory effect of *CsinCPI-2* in periodontal tissues prevented excessive osteoclasts differentiation and bone resorption. *CsinCPI-2* has demonstrated a potential anti-inflammatory and also a pro-osteogenic effect in vitro, increasing the gene expression of the osteogenic markers¹⁰. However, additional studies need to be performed to elucidate the mechanisms through which *CsinCPI-2* promotes its anti-inflammatory effects.

In addition to this indirect effect on osteoclastogenesis, our in vitro results demonstrated that *CsinCPI-2* also has a direct inhibitory effect mechanism on osteoclastogenesis. The results indicated that *CsinCPI-2* is an inhibitor of RANKL-stimulated osteoclast formation by decreasing the TRAP+MuOCL cells and impairing the formation of the actin ring. Similar results have been found with another cystatin, *CysC*; that abolished the formation TRAP+MuOCL and inhibited osteoclastogenesis¹⁶.

In this study, the effect of *CsinCPI-2* inhibiting osteoclast formation was not due to cellular toxicity, but due to the retention of bone marrow macrophages at the macrophage stage. *CsinCPI-2* down-regulated the expression of *Calcr*, *Acp5*, *Nfatc1*, *CtsK* and *Irf8*. These results suggest that *CsinCPI-2* inhibits the expression of RANKL-induced genes involved in osteoclast differentiation and function.

In osteoclastogenesis, RANK and its stimulatory signals with M-CSF for the proliferation and survival of the osteoclast precursor cells are crucially involved⁶².

CsinCPI-2 did not inhibit RANK, but it inhibited the gene expression of *Nfatc1*, which is considered the master regulation in the differentiation of the osteoclast⁶³. When *Nfatc1* is stimulated, it activates the expression of osteoclast specific-genes like *RANK*, *Calcr* and *Trap*, which enables osteoclastogenesis and bone resorption by osteoclasts³⁴⁻⁶⁴.

The results might indicate that *CsinCPI-2* is an interesting drug candidate to control alveolar bone loss associated with PD. Natural products have been the source of many successful drugs and in light of the current absence of a safe and efficient antiresorptive agent to treat PD, *CsinCPI-2* appears as a drug with anti-osteoclastogenic potential.

There are some aspects that our studies did not address. First, the molecular mechanisms by which *CsinCPI-2* targets inflammation and RANK signaling were not evaluated. Furthermore, we used a prophylactic approach to treat mice. It would be interesting to test a therapeutic approach after the onset of disease induction. Finally, the route of administration of *CsinCPI-2* can be further optimized, for instance, a local delivery in nanoparticles or gels, which might benefit the outcomes through reduction of systemic degradation, and increased accessibility of the compound.

7 CONCLUSION

Taken together, our findings indicate that *CsinCPI-2* has the potential to inhibit bone resorption in ligature-induced bone loss and also inhibit osteoclastogenesis in vitro. These outcomes might suggest *CsinCPI-2* as a possible therapeutic candidate to treat inflammatory disorders.

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* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: <http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf>

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APPENDIX A



UNIVERSIDADE ESTADUAL PAULISTA
Faculdade de Odontologia
Câmpus de Araraquara
Comissão de Ética no Uso de Animais - CEUA

CERTIFICADO

Certificamos que o protocolo nº 31/2018 referente à pesquisa **"FITOCISTATINA CSIN-CPI-2, COMO POSSÍVEL CANDIDATO TERAPÊUTICO PARA O TRATAMENTO DE DOENÇAS METABÓLICAS ÓSSEAS"** sob a responsabilidade do **Prof. Dr. Joni Augusto Cirelli** está de acordo com os Princípios Éticos em Experimentação Animal adotado pela legislação brasileira atualmente em vigor, tendo sido aprovado pela Comissão de Ética no Uso de Animais (CEUA) da Faculdade de Odontologia de Araraquara-UNESP.

CERTIFICATE

We certify that the protocolo 31/2018 referring to the research **"PHYTOCYSTATIN CSIN-CPI-2, AS A POTENTIAL THERAPEUTIC CANDIDATE FOR THE TREATMENT OF METABOLIC BONE DISEASES"** under responsibility **Prof. Dr. Joni Augusto Cirelli** in agreement with the nowadays specific brazilian laws and was approved by the Araraquara Dental School-UNESP Ethical Committee for Animal Research (CEUA).

Araraquara, 27 de março de 2020.


Profa. Dra. CARINA APARECIDA FABRÍCIO DE ANDRADE
Coordenador da CEUA/FOAr/UNESP

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Araraquara, 29 de junho de 2021
Natalia Da Ponte Leguizamón