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Periodontal diseases and depression: A pre-clinical in vivo study

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Abstract

Aim: To analyse, through a pre-clinical *in vivo* model, the possible mechanisms linking depression and periodontitis at behavioural, microbiological and molecular levels.

Materials and methods: Periodontitis (P) was induced in Wistar:Han rats (oral gavages with *Porphyromonas gingivalis* and *Fusobacterium nucleatum*) during 12 weeks, followed by a 3-week period of Chronic Mild Stress (CMS) induction. Four groups (n = 12 rats/group) were obtained: periodontitis and CMS (P+CMS+); periodontitis without CMS; CMS without periodontitis; and control. Periodontal clinical variables, alveolar bone levels (ABL), depressive-like behaviour, microbial counts and expression of inflammatory mediators in plasma and brain frontal cortex (FC), were measured. ANOVA tests were applied.

Results: The highest values for ABL occurred in the P+CMS+ group, which also presented the highest expression of pro-inflammatory mediators (TNF-α, IL-1β and NF-kB) in frontal cortex, related to the lipoprotein APOA1-mediated transport of bacterial lipopolysaccharide to the brain and the detection of *F. nucleatum* in the brain parenchyma. A dysregulation of the hypothalamic–pituitary–adrenal stress axis, reflected by the increase in plasma corticosterone and glucocorticoid receptor levels in FC, was also found in this group.

Conclusions: Neuroinflammation induced by *F. nucleatum* (through a leaky mouth) might act as the linking mechanism between periodontal diseases and depression.

KEYWORDS
animal model, depression, *Fusobacterium nucleatum*, inflammation, periodontitis

María Martínez and David Martín-Hernández contributed equally to the work.

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1 | INTRODUCTION

Depression (major depressive disorder) is a common and serious medical illness that causes high medical costs (over 450 billion euros a year in Europe) and negatively affects those who suffer on the way they feel, they think and how they act (Steel et al., 2014).

Its pathophysiology is not completely understood, although it is considered as a multifactorial disease (Kendler et al., 2002), being chronic stress exposure one of the main risk factors, by eliciting a strong effect on the innate immune system (Chrousos & Gold, 1992; Tsigos & Chrousos, 2002; Dudek et al., 2019).

Moreover, other systemic inflammation-related non-communicable diseases have been associated as comorbidities of psychiatric diseases (Cheng et al., 2012; Amare et al., 2017). The biological plausibility of these epidemiological associations has been studied in pre-clinical investigations showing that low-grade inflammation, derived from bacterial translocation, either from the mouth in presence of periodontal diseases ("*leaky mouth*") (Hashioka et al., 2019) or from the gastro-intestinal flora ("*leaky gut*" hypothesis) (Maes et al., 2008; García Bueno et al., 2016), may contribute to the brain pathology associated with neuropsychiatric diseases. This hypothesis has been supported from investigations demonstrating the presence of *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans* in Alzheimer´s post-mortem brains (Emery et al., 2017).

Periodontitis, as a source of chronic low-grade inflammation, has been associated with different mental diseases and neuropsychiatric disorders (Adams et al., 2018; Coelho et al., 2019; Decker et al., 2020), particularly depression (Nascimento et al., 2019), dementia and cognitive decline (Kaye et al., 2010), schizophrenia (Yang et al., 2018) and bipolar disorders (Cunha et al., 2019). However, in spite of these epidemiologic and in vivo experimental evidence (Breivik et al., 2006), there is limited evidence on the mechanisms by which periodontitis might influence the physiopathology of depression. It was, therefore, the objective of this *in vivo* pre-clinical investigation to study the inflammatory-related mechanisms that may link periodontitis and depressive symptoms. The specific objectives were (a) to develop an experimental model to study the influence of periodontitis in psychiatric diseases, (b) to characterize the inflammatory response at a molecular level and the depressive-like behaviour induced by the combined exposure to periodontitis and depression and (c) to analyse the presence of *P. gingivalis* and *Fusobacterium nucleatum* in brain tissues as potential triggers of inflammation.

Clinical Relevance

Scientific rationale for study: In the last years, the role of neuroinflammation in the pathophysiology of major depression has been highlighted. In absence of a clear infection, the state of chronic low-grade systemic inflammation characteristic of periodontitis may contribute to neuroinflammation.

Principal findings: Increased gingival inflammation, alveolar bone loss and inflammatory response, in rats' plasma and brain, were found in the combined model of periodontitis and CMS.

Practical implications: Neuroinflammation seems to be higher in animals included in the combined model. The management of periodontitis might be included in the preventive armamentarium against mood disorders, including major depression.

2 | MATERIAL AND METHODS

This pre-clinical *in vivo* study was designed according to the modified ARRIVE guidelines for pre-clinical research (Vignoletti & Abrahamsson, 2012) and following the Spanish and European Union regulations (European Communities Council Directive 86/609/EEC) (Appendix 1). Male Wistar Hannover rats (HsdRccHan:Wist, Envigo, Spain) (230–250 g) were kept in constant conditions for 7 days prior to experiments (Appendix 2).

2.1 | Study design

The study design is reflected in Figure 1. Four experimental groups resulted from the different combinations of periodontitis (P) and chronic mild stress (CMS) induction: (a) periodontitis group (P+CMS−); (b) periodontitis and CMS group (P+CMS+); (c) CMS group (P−CMS+); and (d) control group (P−CMS−).

In phase 1 (12 weeks), periodontitis was induced using the oral gavages method, and four animals were housed in each cage. In phase 2 (3 weeks), the depressive-like behaviour model was induced by exposing the rats to CMS, and animals were isolated.

The experimental periodontitis model (Virto et al., 2018) consisted on inoculating *P. gingivalis* ATCC W83 K1 and *F. nucleatum* DMSZ 20482 through oral gavages. These bacteria were grown individually in anaerobic conditions (80% N_2, 10% H_2, 10% CO_2 at 37°C) in brain heart infusion (BHI) media (25 ml) (Becton, Dickinson and Company). Bacterial growth was adjusted by spectrophotometry at 550 nm to obtain 10^9 colonies forming units (CFU)/ml for each bacterium. Both pure cultures were mixed and centrifuged (10 min at 1,520 *g*) in order to separate the bacteria from the culture medium. The precipitated bacteria were re-suspended in 50 ml of sterile gavages solution [phosphate-buffered saline (PBS) at 2% of carboxymethyl cellulose (Sigma)]. The gavage solution without bacteria was used as a placebo. One millilitre of the suspension was administered in four consecutive days per week over 12 weeks, with a sterile insulin syringe without needle, early in the morning taking the animals out of the cage, always in the same order (P−CMS+, P−CMS−, P+CMS+ and P+CMS−), to avoid bacterial contamination.

The experimental depressive-like behaviour model (Garate et al., 2011; Martin-Hernandez et al., 2016) was an adaptation from the CMS proposed by Willner, (2005). It consisted on introducing a series of different stressors that are changed daily (two stressors/day). They are given in an unpredictable basis for a period of 21 days plus

one extra day to maintain the stress exposure during the behavioural tests. These stressors included: (a) food deprivation, (b) water deprivation, (c) cage tilting, (d) soiled cage, (e) grouped housing after a period of water deprivation, (f) stroboscopic illumination (150 flashes/min) and (g) intermittent illumination every 2 h.

2.1.1 | Tissue specimens

At the end of phase 2, rats were sacrificed by terminal anaesthesia using sodium pentobarbital (320 mg/kg i.p. Vetoquinol®, Madrid, Spain) followed by decapitation. Samples of blood, mandible and brain were harvested. To avoid the possible influence of the circadian rhythm, the collection of biological samples was always done at the same time (namely between 2 and 3 PM). Blood samples were collected by cardiac puncture, then anti-coagulated with ethylenediaminetetraacetic acid (EDTA, 1% w:v, 1 vol EDTA per 50 vol blood) and centrifuged at 366 *g* and room temperature for 15 min to obtain plasma. The mandible and brain were removed from the skull. The brain was divided into hemispheres through the inter-hemispheric fissure, using the whole right side for microbiological detection and the left frontal cortex (FC) for inflammatory mediator analyses. All these specimens were immediately frozen at −80°C.

2.2 | Study outcomes

2.2.1 | Periodontal outcomes

Periodontal clinical variables were recorded at the first molars, with the rats under anaesthesia using a mixture of ketamine (0.08 ml/100 g)/Xylazine (0.04 ml/100 g). One trained examiner (MM) recorded the following clinical variables at baseline, and at 12 (post-periodontitis induction) and 15 (post-CMS) weeks: modified gingival index (GI) (Lobene et al., 1986), probing depth (PD) and bleeding on probing (BOP) with the use of a 0.4 mm round-ended probe (UNC12, Hu-Friedy, Mfg. Co., LLC, Chicago, USA) under magnification (×3.4).

Alveolar bone levels (ABL) were measured from one hemi-mandible per animal once the soft tissues were eliminated. Blue-methylene staining was used to facilitate the location of the cemento-enamel junction (CEJ) and direct measurements to the alveolar crest were made using a Nikon SMZ800 microscope at 1.5× magnification. The Leica Application Suite was used to obtain different linear measurements (Figure 2).

2.2.2 | Microbiological outcomes

Bacterial DNA was extracted from blood samples and half brains using specifically designed commercial kits: MoIYsis Complete5 and Ultra-Deep Microbiome Prep, respectively (Molzym Gmbh& Co.KG.). The extracted DNA was then eluded in 100 µl of sterile water (Roche) and frozen at −20°C for further analysis. These DNA samples were analysed with a previously validated quantitative polymerase chain reaction test (qPCR), based on detecting highly specific 16S rRNA genes of *P. gingivalis* and *F. nucleatum* (Marin et al., 2018; Marin et al., 2019) (Appendix 3).

2.2.3 | Evaluation of depressive-like behaviour

Body weight was measured at the beginning of each week during the CMS protocol to control the effects of the stress protocol and as a physiological assessment. In addition, three behavioural tests were performed to evaluate anxiety and anhedonia at day 21 of the CMS protocol: elevated plus maze (Pellow et al., 1985), splash test (Yalcin et al., 2005) and sucrose preference/consumption test (Grippo et al., 2005). Detailed information is presented in Appendix 4.

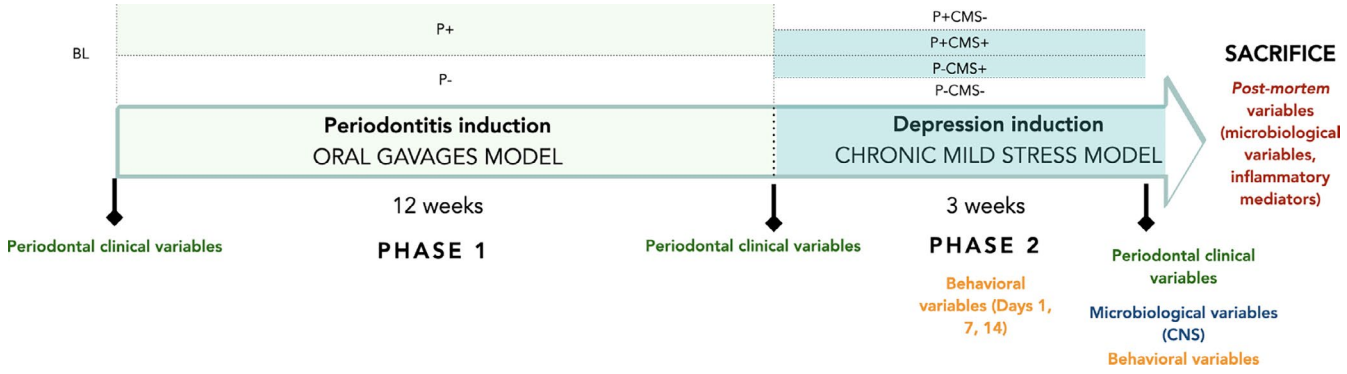

FIGURE 1 Study design. The study design consists in two phases: periodontitis induction (phase 1, 12 weeks) and depression induction (CMS exposure) (phase 2, 3 weeks). In phase 1, half of the animals received oral gavages (P+). Four experimental groups resulted after these two phases: (1) periodontitis group (P+CMS−); (2) periodontitis and CMS group (P+CMS+); (3) CMS group (P−CMS+) and (4) control group (P−CMS−). Periodontal clinical variables were registered at baseline, and after phase 1 and 2. Behavioural variables were registered during and after phase 2. After sacrifice, post-mortem variables, including microbiological and inflammatory variables, were analysed. BL, Baseline; CMS, chronic mild stress; P, periodontitis

2.2.4 | Inflammatory mediators

The quantification of inflammatory mediators was performed in plasma and/or FC following different techniques (Appendix 5):

a. Nuclear extraction and total homogenate procedures

Using a method that provides a high purity nuclear fraction (Garate et al., 2011) (Appendix 6).

b. Western blot analysis

To determine the protein expression of glucocorticoid receptor (GR) and p65 subunit of NF-κB (p65) using nuclear extracts from FC samples. Toll-like receptor-4 (TLR-4), inducible NO synthase (iNOS), phospho-p38 mitogen-activated protein kinase (p-p38), α and β subunits of p38 (p38 α/β), apolipoprotein A-1 (APO A1), phospho-mammalian target of rapamycin (p-mTOR) and m-TOR were analysed in the FC total homogenates (Appendix 7).

c. Protein assay

Protein levels were measured using the Bradford method based on the principle of protein-dye binding.

d. Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR analyses were carried out homogenizing FC in 500 μl of TRIZOL® reagent (Invitrogen, Life Technologies) in the TissueLyser LT (QUIAGEN®). The frequency used was 50 oscillations per second for 5 min at 4°C. Total cytoplasmic RNA was prepared from samples following TRIZOL® datasheet; aliquots were converted to cDNA by reverse transcription using random hexamer primers. Primer oligonucleotides for PCR were designed with the Primer3 tool (Untergasser et al., 2012). Target specificity was checked by in silico PCR using the USCS GenomeBrowser (Kent et al., 2002) and Blast (NCBI) for cDNA and gDNA; only primer pairs with no unintended targets were selected. Semi-quantitative changes in mRNA levels were estimated by RT-PCR using specific conditions (Appendix 8).

e. Plasma corticosterone, lipopolysaccharide and lipopolysaccharide binding protein

Corticosterone levels were assayed employing a commercially available ELISA (ENZO Life Sciences) following the manufacturer's instructions. Plasma lipopolysaccharide (LPS) and lipopolysaccharide binding protein (LBP) levels were determined using commercially available kits following the manufacturer's instructions (Hycult Biotech).

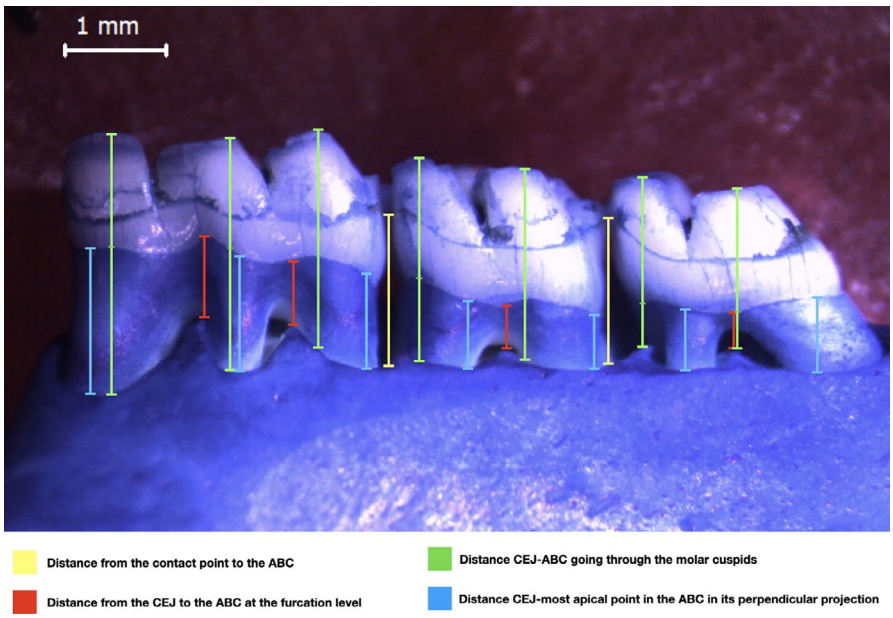

FIGURE 2 Description of the variables used in the morphometric analysis variables to evaluate alveolar bone levels. Alveolar bone levels were obtained by direct linear measurement from different anatomical landmarks: (1) distance from the contact points to the alveolar bone crest in its perpendicular projection (represented by a yellow line); (2) distance from the cemento-enamel junction to the alveolar bone crest at the furcation level (represented by a red line); (3) distance cemento-enamel junction and alveolar bone crest going through the molar cuspids (represented by a green line); (4) distance from the cemento-enamel junction to the most apical point of the alveolar bone crest in its perpendicular projection (represented by a blue line). ABC, alveolar bone crest; CEJ, cemento-enamel junction

TABLE 1 Periodontal outcomes: (a) Clinical outcomes; (b) Morphometric analyses

Table 1a

							Post hoc comparison					
									95% CI			
			n	Mean	SD	Global *p*-value (ANOVA)	Specific comparison	Mean difference	Lower Bound	Upper Bound	Post hoc *p*-value (ANOVA)	Post hoc *p*-value (GLM)
Periodontal clinical outcomes												
GI	Baseline	P+CMS−	12	0.03	0.04	.154	P+CMS− vs. P+CMS+	−0.01	−0.07	0.04	1.000	1.000
		P+CMS+	12	0.04	0.06		P+CMS− vs. P−CMS+	−0.01	−0.07	0.04	1.000	1.000
		P−CMS+	12	0.04	0.07		P+CMS− vs. P−CMS−	0.03	−0.03	0.08	1.000	1.000
		P−CMS−	11	0.00	0.00		P+CMS+ vs. P−CMS+	0.00	−0.05	0.05	1.000	1.000
							P+CMS+ vs. P−CMS−	0.04	−0.01	0.10	.278	.113
							P−CMS+ vs. P−CMS−	0.04	−0.01	0.10	.278	.817
	Post-periodontitis induction	P+CMS−	10	0.38	0.19	**<.001**	P+CMS− vs. P+CMS+	−0.15	−0.36	0.06	.343	.388
		P+CMS+	11	0.52	0.23		P+CMS− vs. P−CMS+	0.31	0.11	0.52	**.001**	**.001**
		P−CMS+	12	0.06	0.09		P+CMS− vs. P−CMS−	0.18	−0.03	0.40	.135	.153
		P−CMS−	10	0.19	0.16		P+CMS+ vs. P−CMS+	0.46	0.26	0.66	**<.001**	**<.001**
							P+CMS+ vs. P−CMS−	0.33	0.12	0.54	**<.001**	**.001**
							P−CMS+ vs. P−CMS−	−0.13	−0.33	0.08	.529	.478
	Post-CMS induction	P+CMS−	11	0.69	0.30	**.001**	P+CMS− vs. P+CMS+	−0.04	−0.33	0.26	1.000	1.000
		P+CMS+	11	0.73	0.29		P+CMS− vs. P−CMS+	0.28	−0.01	0.57	.069	.166
		P−CMS+	11	0.41	0.22		P+CMS− vs. P−CMS−	0.36	0.05	0.66	**.013**	**.036**
		P−CMS−	10	0.33	0.15		P+CMS+ vs. P−CMS+	0.32	0.02	0.61	**.027**	**.037**
							P+CMS+ vs. P−CMS−	0.39	0.09	0.70	**.005**	**.007**
							P−CMS+ vs. P−CMS−	0.08	−0.23	0.38	1.000	1.000
PD	Baseline	P+CMS−	12	0.12	0.01	.077	P+CMS− vs. P+CMS+	−0.01	−0.02	0.01	1.000	1.000
		P+CMS+	12	0.12	0.02		P+CMS− vs. P−CMS+	0.00	−0.01	0.02	1.000	1.000
		P−CMS+	12	0.11	0.01		P+CMS− vs. P−CMS−	0.01	−0.01	0.02	1.000	1.000
		P−CMS−	11	0.11	0.01		P+CMS+ vs. P−CMS+	0.01	0.00	0.03	.183	.663
							P+CMS+ vs. P−CMS−	0.01	0.00	0.03	.124	.479

Table 1a

							Post hoc comparison					
									95% CI			
			n	Mean	SD	Global *p*-value (ANOVA)	Specific comparison	Mean difference	Lower Bound	Upper Bound	Post hoc *p*-value (ANOVA)	Post hoc *p*-value (GLM)
							P−CMS+ vs. P−CMS−	0.00	−0.01	0.02	1.000	1.000
	Post-periodontitis induction	P+CMS−	10	0.26	0.07	**<.001**	P+CMS− vs. P+CMS+	0.05	0.00	0.10	.094	.092
		P+CMS+	11	0.21	0.04		P+CMS− vs. P−CMS+	0.14	0.09	0.20	**<.001**	**<.001**
		P−CMS+	12	0.12	0.02		P+CMS− vs. P−CMS−	0.14	0.08	0.19	**<.001**	**<.001**
		P−CMS−	10	0.13	0.03		P+CMS+ vs. P−CMS+	0.10	0.04	0.15	**<.001**	**<.001**
							P+CMS+ vs. P−CMS−	0.09	0.03	0.14	**<.001**	**<.001**
							P−CMS+ vs. P−CMS−	−0.01	−0.06	0.04	1.000	1.000
	Post-CMS induction	P+CMS−	11	0.29	0.08	**<.001**	P+CMS− vs. P+CMS+	0.07	0.01	0.14	**.008**	**.003**
		P+CMS+	12	0.21	0.05		P+CMS− vs. P−CMS+	0.11	0.05	0.17	**<.001**	**<.001**
		P−CMS+	11	0.18	0.03		P+CMS− vs. P−CMS−	0.14	0.08	0.21	**<.001**	**<.001**
		P−CMS−	10	0.15	0.02		P+CMS+ vs. P−CMS+	0.04	−0.02	0.10	.600	1.000
							P+CMS+ vs. P−CMS−	0.07	0.01	0.13	**.024**	.055
							P−CMS+ vs. P−CMS−	0.03	−0.03	0.09	1.000	1.000
BOP	Baseline	P+CMS−	12	0.00	0.00	.595	P+CMS− vs. P+CMS+	−0.01	−0.03	0.01	1.000	1.000
		P+CMS+	12	0.01	0.02		P+CMS− vs. P−CMS+	−0.01	−0.03	0.01	1.000	1.000
		P−CMS+	12	0.01	0.02		P+CMS− vs. P−CMS−	0.00	−0.02	0.02	1.000	1.000
		P−CMS−	11	0.00	0.00		P+CMS+ vs. P−CMS+	0.00	−0.02	0.02	1.000	1.000
							P+CMS+ vs. P−CMS−	0.01	−0.01	0.03	1.000	1.000
							P−CMS+ vs. P−CMS−	0.01	−0.01	0.03	1.000	1.000
	Post-periodontitis induction	P+CMS−	10	0.07	0.08	**.007**	P+CMS− vs. P+CMS+	−0.01	−0.09	0.07	1.000	1.000
		P+CMS+	11	0.08	0.10		P+CMS− vs. P−CMS+	0.07	−0.01	0.14	.104	.123
		P−CMS+	12	0.00	0.00		P+CMS− vs. P−CMS−	0.07	−0.01	0.15	.134	.140
		P−CMS−	10	0.00	0.00		P+CMS+ vs. P−CMS+	0.08	0.00	0.15	**.040**	**.029**
							P+CMS+ vs. P−CMS−	0.08	0.00	0.15	.056	**.035**

Table 1a

						Post hoc comparison					
								95% CI			
		n	Mean	SD	Global *p*-value (ANOVA)	Specific comparison	Mean difference	Lower Bound	Upper Bound	Post hoc *p*-value (ANOVA)	Post hoc *p*-value (GLM)
Post-CMS induction	P+CMS−	11	0.10	0.16	. 374	P−CMS+ vs. P−CMS−	0.00	−0.08	0.08	1.000	1.000
						P+CMS− vs. P+CMS+	0.04	−0.09	0.16	1.000	1.000
	P+CMS+	11	0.06	0.08		P+CMS− vs. P−CMS+	0.06	−0.06	0.18	1.000	1.000
	P−CMS+	11	0.04	0.09		P+CMS− vs. P−CMS−	0.07	−0.05	0.20	.652	.704
	P−CMS−	10	0.03	0.06		P+CMS+ vs. P−CMS+	0.03	−0.10	0.15	1.000	1.000
						P+CMS+ vs. P−CMS−	0.04	−0.09	0.16	1.000	1.000
						P−CMS+ vs. P−CMS−	0.01	−0.11	0.14	1.000	1.000

Table 1b

					Post hoc comparison				
							95% Confidence Interval for mean		
	n	Mean	SD	Global *p*-value (ANOVA)	Specific comparison	Mean Difference	Lower Bound	Upper Bound	Post hoc *p*-value (ANOVA)
Distance CEJ-ABC at the furcation level									
P+CMS−	8	0.55	0.08	**.039**	P+CMS− vs. P+CMS+	−0.12	−0.25	0.01	.097
P+CMS+	8	0.67	0.07		P+CMS− vs. P−CMS+	−0.01	−0.15	0.12	1.000
P−CMS+	8	0.56	0.13		P+CMS− vs. P−CMS−	0.02	−0.14	0.17	1.000
P−CMS-	5	0.53	0.08		P+CMS+ vs. P−CMS+	0.11	−0.03	0.24	.184
					P+CMS+ vs. P−CMS−	0.13	−0.02	0.29	.104
					P−CMS+ vs. P−CMS−	0.03	−0.12	0.18	1.000
Distance contact point-ABC									
P+CMS−	8	0.99	0.11	.215	P+CMS− vs. P+CMS+	−0.16	−0.39	0.07	.371
P+CMS+	9	1.14	0.15		P+CMS− vs. P−CMS+	−0.14	−0.38	0.10	.631
P−CMS+	8	1.13	0.23		P+CMS− vs. P−CMS−	−0.06	−0.31	0.20	1.000
P−CMS-	6	1.04	0.14		P+CMS+ vs. P−CMS+	0.02	−0.21	0.25	1.000
					P+CMS+ vs. P−CMS−	0.10	−0.15	0.35	1.000
					P−CMS+ vs. P−CMS−	0.08	−0.17	0.34	1.000

Table 1b

					Post hoc comparison				
							95% Confidence Interval for mean		
	n	Mean	SD	Global *p*-value (ANOVA)	Specific comparison	Mean Difference	Lower Bound	Upper Bound	Post hoc *p*-value (ANOVA)
Distance CEJ-ABC at the cuspid level									
P+CMS−	8	1.95	0.08	.290	P+CMS− vs. P+CMS+	−0.08	−0.25	0.08	.972
P+CMS+	9	2.03	0.16		P+CMS− vs. P−CMS+	−0.05	−0.22	0.13	1.000
P−CMS+	8	2.00	0.14		P+CMS− vs. P−CMS−	0.03	−0.15	0.20	1.000
P−CMS−	7	1.92	0.07		P+CMS+ vs. P−CMS+	0.04	−0.13	0.21	1.000
					P+CMS+ vs. P−CMS−	0.11	−0.06	0.28	.487
					P−CMS+ vs. P−CMS−	0.07	−0.11	0.25	1.000
Distance CEJ-ABC in its most apical point									
P+CMS−	8	0.74	0.10	.410	P+CMS− vs. P+CMS+	−0.08	−0.22	0.05	.583
P+CMS+	9	0.83	0.12		P+CMS− vs. P−CMS+	−0.05	−0.20	0.09	1.000
P−CMS+	8	0.80	0.10		P+CMS− vs. P−CMS−	−0.05	−0.19	0.10	1.000
P−CMS−	7	0.79	0.06		P+CMS+ vs. P−CMS+	0.03	−0.11	0.17	1.000
					P+CMS+ vs. P−CMS−	0.04	−0.11	0.18	1.000
					P−CMS+ vs. P−CMS−	0.01	−0.14	0.15	1.000

Abbreviations: ABC, alveolar bone crest; BOP, bleeding on probing; CEJ, cemento-enamel junction; CI, confidence interval; CMS, chronic mild stress; CMS, chronic mild stress, GLM, general lineal model; GI, gingival index; P, periodontitis; P, periodontitis; PD, probing depth; SD, standard deviation. Bold values indicate *p* values ≤ 0.05.

2.3 | Data analyses

Due to the lack of previous studies, previous data using the CMS model were used to detect a difference of 1.6 × sigma in the expression of inflammatory parameters at protein and mRNA levels with a hypothetical standard deviation (SD) of 25 (Martin-Hernandez et al., 2016). With these parameters, the resulting sample size was 10 animals per group. In order to compensate for potential drop-outs, two more animals per group were selected (n = 48; 12 per group).

The animal was considered the unit of analyses, and the outcomes were calculated by animal and then per group, expressing data as means and SD. For quantitative variables, normality of the distribution was evaluated with box plots and Shapiro–Wilk test. In case the data were not normally distributed, variables were log-transformed.

Central nervous system (CNS) and plasma variables were checked for inhomogeneity of variances by Brown–Forsythe test. Whenever SD were different, a Brown–Forsythe ANOVA test followed by a Tamhane's T2 for multiple comparisons were run. In order to analyse intergroup differences, ANOVA tests with post hoc Bonferroni corrections (periodontal variables) and Tukey (CNS and plasma variables) were used. In addition, repeated measures ANOVA with Bonferroni corrections were used for the clinical periodontal outcomes. Categorical variables were analysed with chi-squared test.

A p-value ≤ .05 was defined as statistically significant. All analyses were carried out using IBM SPSS Statistics 25.0.0.0 (IBM) or GraphPad Prism© version 7.00. (GraphPad Software, Inc.).

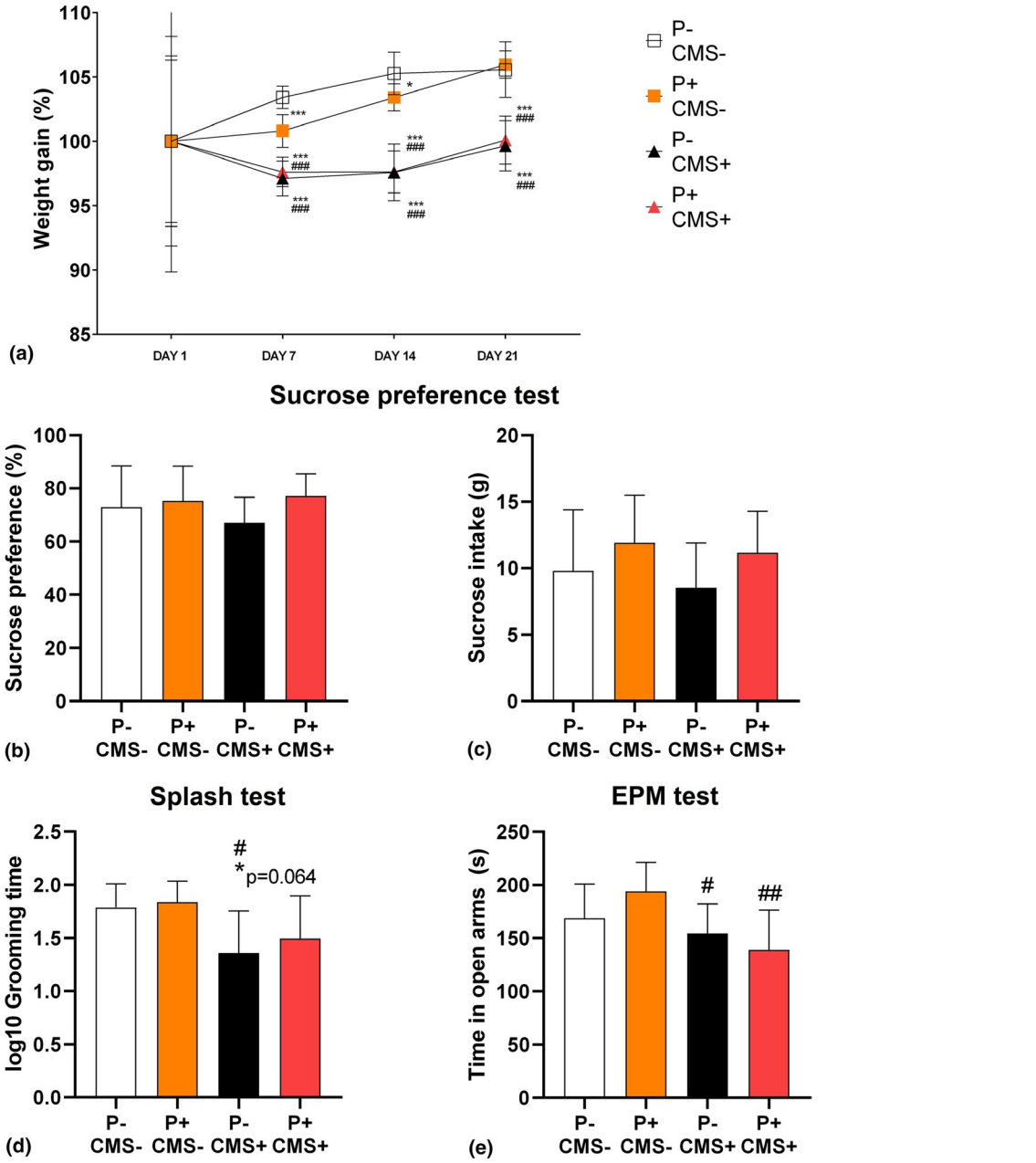

FIGURE 3 Evaluation of depressive-like behaviour after periodontitis induction (P), chronic mild stress exposure (CMS), and both protocols combined (P+CMS+). Percentage of weight gain at days 1, 7, 14 and 21 of the CMS protocol (a); sucrose preference (b), and sucrose intake (c) (b, c are parameters of the sucrose preference test), splash test (d), and elevated plus maze (EPM) (e). Data are means ± SD of 8–12 rats per group; *$p < 0.05$ versus P−CMS−; ***$p < 0.001$ versus P−CMS−; #$p < 0.05$ versus P+CMS−; ##$p < 0.01$ versus P+CMS−; ###$p < 0.001$ versus P+CMS−. One-way ANOVA with a Tukey post hoc for day 7 of the % weight, sucrose preference, sucrose intake, splash test and EPM. Brown–Forsythe ANOVA test with a Tamhane's T2 for days 1, 14, and 21 of the % weight

3 | RESULTS

3.1 | Study sample

The study flow diagram is presented in Appendix 9. Initially, 12 animals were allocated per group. Two rats died at baseline (P-CMS- group) and two after periodontitis induction (P+CMS− and P−CMS+ groups), during the administration of the anaesthesia for recording the clinical outcomes. Immediately after euthanasia, three rats per group were perfused with paraformaldehyde for further immunohistochemistry studies, and therefore, their data were not included in this manuscript. Any other change in the sample sizes in biochemical or behavioural determinations was due to methodological pitfalls.

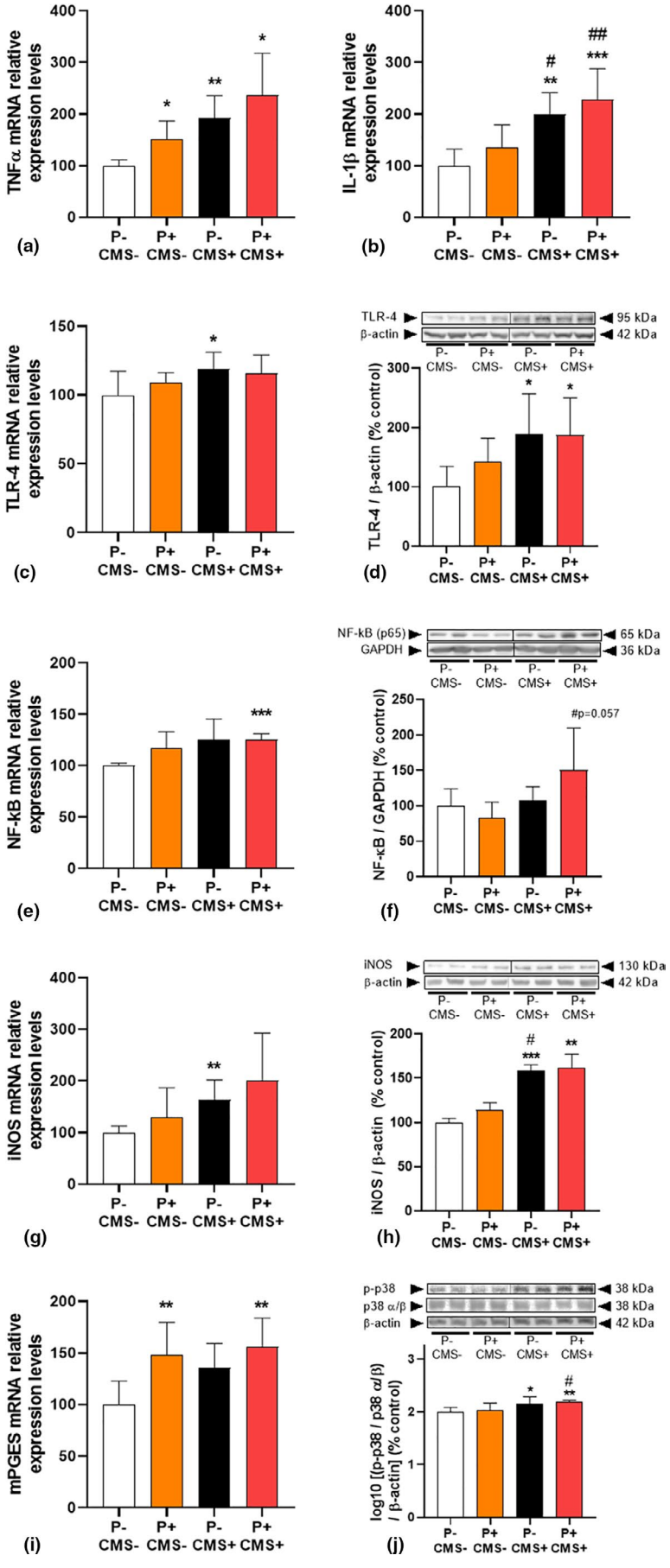
TNFα mRNA relative expression levels
(a)
IL-1β mRNA relative expression levels
(b)
TLR-4 mRNA relative expression levels
(c)
TLR-4
β-actin
95 kDa
42 kDa
TLR-4 / β-actin (% control)
(d)
NF-kB mRNA relative expression levels
(e)
NF-kB (p65)
GAPDH
65 kDa
36 kDa
#p=0.057
NF-κB / GAPDH (% control)
(f)
iNOS mRNA relative expression levels
(g)
iNOS
β-actin
130 kDa
42 kDa
iNOS / β-actin (% control)
(h)
mPGES mRNA relative expression levels
(i)
p-p38
p38 α/β
β-actin
38 kDa
38 kDa
42 kDa
log10 [(p-p38 / p38 α/β) / β-actin] (% control)
(j)
P- CMS-
P+ CMS-
P- CMS+
P+ CMS+

FIGURE 4 Quantification of inflammatory mediators in frontal cortex brain samples after periodontitis induction (P), chronic mild stress exposure (CMS), and both protocols combined (P+CMS+). Messenger ribonucleic acid (mRNA) expression in the frontal cortex of the pro-inflammatory cytokines tumour necrosis factor-alpha (TNFα) (a) and interleukin-1 beta (IL-1β) (b), the innate immune toll-like receptor 4 (TLR-4) (c), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (e), inducible nitric oxide synthase (iNOS) (g), and microsomal prostaglandin E synthase (mPGES) (i). Protein expression in the frontal cortex of TLR-4 (d), NF-kB in nuclear fraction (f), iNOS (h), and phosphorylation ratio of p38 mitogen-activated protein kinase (p-p38/p38α/β) (j). Blots were cropped (black lines) for improving the clarity and conciseness of the presentation. The densitometric data of the band of interest were normalized by beta-actin (β-actin), except for the nuclear expression of NF-kB which was normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The mRNA expression was normalized by beta-tubulin (β-tubulin). Data are means ± SD of 6–9 rats per group; $^{*}p < 0.05$ versus P−CMS−; $^{**}p < 0.01$ versus P−CMS−; $^{***}p < 0.001$ versus P−CMS−; $\#p < 0.05$ versus P+CMS−; $\#\#p < 0.01$ versus P+CMS−. One-way ANOVA with a Tukey post hoc for IL-1β mRNA, TLR-4 mRNA, TLR-4 protein, mPGES mRNA, and p-p38 ratio protein. Brown−Forsythe ANOVA test with a Tamhane's T2 for TNFα mRNA, NF-kB mRNA, NF-kB nuclear protein, iNOS mRNA, and iNOS protein

3.2 | Periodontal outcomes

At baseline, there were no statistically significant differences among groups in any periodontal clinical outcome (Table 1). After periodontitis induction, groups that underwent periodontitis induction (P+) showed significantly ($p < 0.001$) higher PD and GI, when compared with P− groups. After inducing CMS, P+CMS+ demonstrated the highest GI values (0.73 [SD = 0.29]), followed by P+CMS− (0.69 [SD = 0.30]). Mean PD were similar in P+ groups, P+CMS− and P+CMS+ [0.29 (SD = 0.08) mm and 0.21 (SD = 0.05) mm, respectively].

P+CMS+ showed the highest ABL measurements at the level of the furcation [0.67 (SD = 0.07) mm] and at the level of the cuspid [2.03 (SD = 0.16) mm]. Statistically significant differences among the groups were found at the level of the furcation ($p = .039$).

3.3 | Microbiological outcomes

Porphyromonas gingivalis could not be found either in brain or in blood. *Fusobacterium nucleatum* was found in the brain of two rats in the P+CMS+ group [24.3 colony-forming units (CFU)/mL; SD = 46.27)], and in blood from one rat in P+CMS+ and in two control rats.

3.4 | Evaluation of depressive-like behaviour

Both CMS+ groups showed a significant reduced weight at days 7, 14 and 21 of stress. No significant differences between groups were found in the sucrose preference test ($p = .183$), although there was a tendency for more sucrose intake in the groups P+ compared to P− (Figure 3 and Appendix 10). In the splash test, P−CMS+ animals spent less grooming time than both P−CMS− ($p = .064$) and P+CMS− groups. Finally, both P−CMS+ and P+CMS+ groups spent less time in open arms compared to P+CMS− rats.

3.5 | Quantification of inflammatory mediators in frontal cortex

The highest mRNA levels of tumour necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β) were found in P+CMS+ (Figure 4 and Appendix 11). Regarding TLR4 signalling pathway, P+CMS+ group presented increased protein levels of TLR4, iNOS and p-p38 compared to P−CMS−, along with upregulated mRNA expression of NF-kB and mPGES. An increase in NF-kB nuclear protein levels compared to P+CMS− was also detected ($p = .057$).

In the P−CMS+ group, TNF-a, IL-1b, TLR-4, iNOS and p-p38 were upregulated compared to P−CMS−, but no changes on NF-kB and mPGES were found.

A possible origin and consequences of neuroinflammation are reported in Figure 5 and Appendix 12. A significant decrease was found in LPS plasma levels in P+CMS+ compared to controls and P+CMS−. LBP, one of the LPS plasma transport proteins, was also downregulated in P+CMS+ in comparison with the other experimental groups. APOA1 protein expression, with potentially binding for LPS and transport to the CNS, was increased in the P−CMS+ and P+CMS+ animals compared to both control and P+CMS− groups.

An increase in plasma corticosterone levels in P+CMS+, compared to controls, and in GR expression in the FC of P+CMS+ rats, compared to P+CMS− and P−CMS+, were observed. The ratio of p-mTOR/m-TOR was decreased in P+CMS+ compared to P+CMS−.

4 | DISCUSSION

The main findings of the present *in vivo* pre-clinical investigation are shown in Figure 6: the selected experimental model was able to induce periodontitis, neuroinflammation and a depressive-like behaviour. Animals in which periodontitis was induced showed increased periodontal pathology, as demonstrated by significantly higher values in periodontal clinical outcomes. Similarly, animals that received CMS evidenced a depressive-like behaviour in terms of a decrease in weight gain and grooming, and a higher time in open arms in the EPM test (anhedonia and anxiety). The combination of periodontitis and CMS induction resulted in higher values in periodontal outcomes as well as in inflammatory mediators in the brain (TNF-α, IL-1β and NF-kB). Although LPS and LBP plasma levels were lower in P+CMS+ rats, the increase of APOA1 in FC induced by CMS could suggest a mechanism of LPS transport to CNS, contributing to the detected neuroinflammation (Vargas-Caraveo et al., 2017). This experimental model also evidenced the presence of *F. nucleatum* in the brain tissue of two rats from the P+CMS+ group, which may be also considered as a source of neuroinflammation, that could be directly related

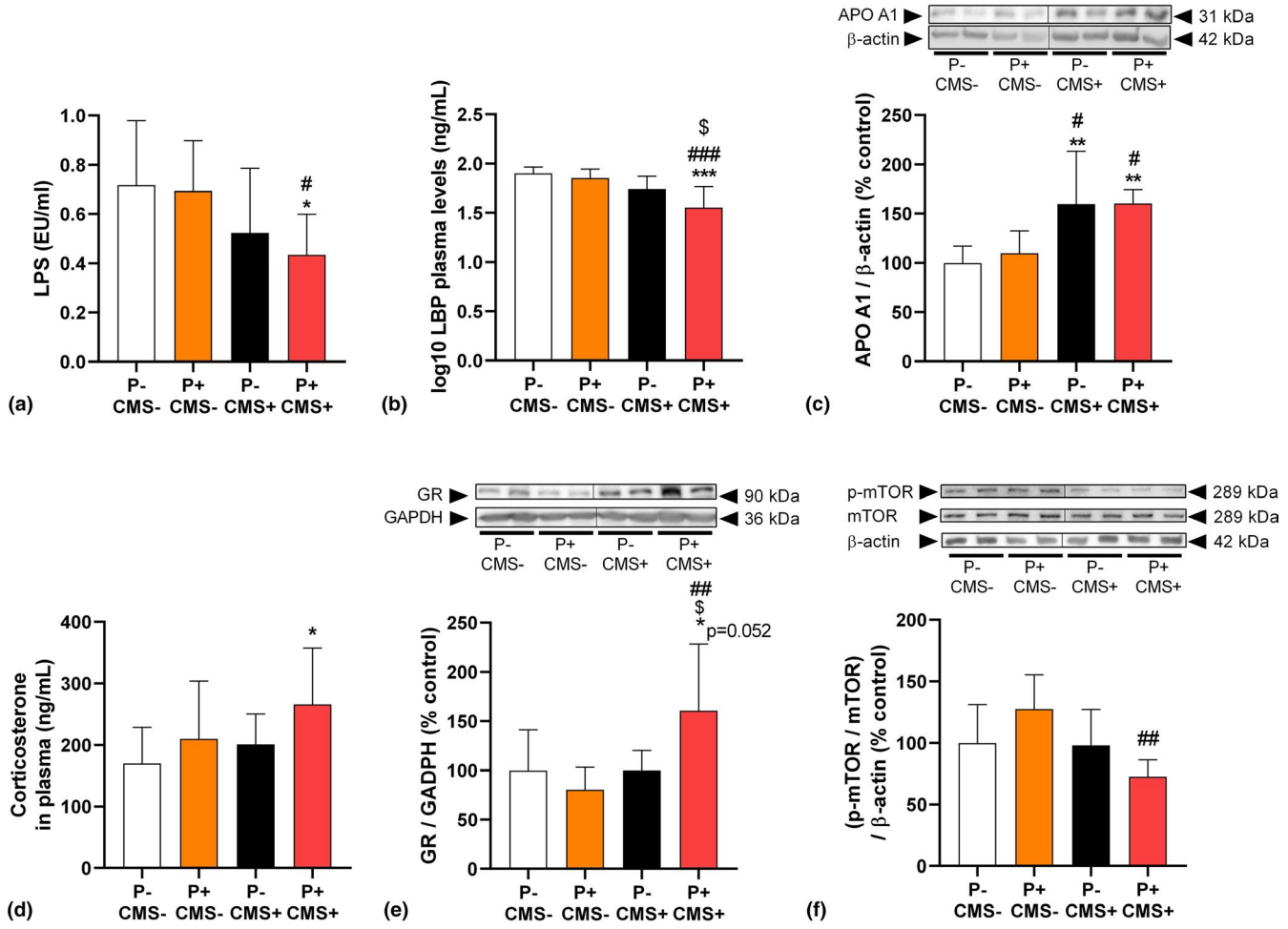

FIGURE 5 Possible origin and consequences of neuroinflammation after periodontitis induction (P), chronic mild stress exposure (CMS), and both protocols combined (P+CMS+). Lipopolysaccharide (LPS) endotoxin units (EU) in plasma (a), lipopolysaccharide binding protein (LBP) plasma levels (b), apolipoprotein-A1 (APO-A1) protein expression in the frontal cortex (c), corticosterone plasma levels (d), glucocorticoid receptor (GR) nuclear protein expression in the frontal cortex (e), and phosphorylation ratio of mammalian target of rapamycin (m-TOR / m-TOR) in the frontal cortex (f). Blots were cropped (black lines) for improving the clarity and conciseness of the presentation. The densitometric data of the band of interest were normalized by beta-actin (β-actin), except for the nuclear expression of GR which was normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are means ± SD of 7–12 rats per group; *$p < 0.05$ versus P-CMS-; **$p < 0.01$ versus P-CMS-; ***$p < 0.001$ versus P–CMS–; #$p < 0.05$ versus P+CMS–; ##$p < 0.01$ versus P+CMS–; ###$p < 0.001$ versus P+CMS–; $$p < 0.05$ versus P–CMS+. One-way ANOVA with a Tukey post hoc

to the dysregulation of the hypothalamic–pituitary–adrenal (HPA) stress axis, reflected by the increase in plasma corticosterone and GR levels in FC in the P+CMS+ group, and the decline in p-mTOR activation that could affect cellular survival.

The oral gavage with periodontal pathogens periodontitis induction model was selected, based on the positive results reported in previous studies (Polak et al., 2009; Virto et al., 2018), in spite of the ligature periodontitis induction model being the most widely used (Takada et al., 2004; Breivik et al., 2006; Araujo et al., 2017). In fact, placement of ligatures has demonstrated more severe periodontal destruction in shorter time, when compared with oral gavages with periodontopathogens (de Molon et al., 2016), but ligatures always exert a high degree of trauma to the periodontal tissues (Klausen, 1991) and the oral gavage method has previously shown periodontal bacterial translocation to the blood flow (Castillo et al., 2011; Figuero et al., 2014). Male rats were selected to avoid any possible hormonal fluctuation related to periodontitis (Tatakis & Trombelli, 2004).

In regard to depression induction, also other models, such as olfactory bulbectomy, have been used (Breivik et al., 2006). However, CMS has a higher translational potential (Willner, 2017) and has been extensively used in the study of neuroinflammation (Garate et al., 2011; Wang et al., 2018).

The possible mechanisms underlying the bidirectional association between depression and periodontitis have been suggested (Breivik et al., 1996; Boyapati & Wang, 2007), including that periodontitis could be associated with depression via neuroinflammation. In fact, the present study has shown that the P+CMS+ group showed the greatest values of inflammatory mediators (Il-1β, TNF-α, NF-κB) in FC brain samples. Moreover, stress exposure has been

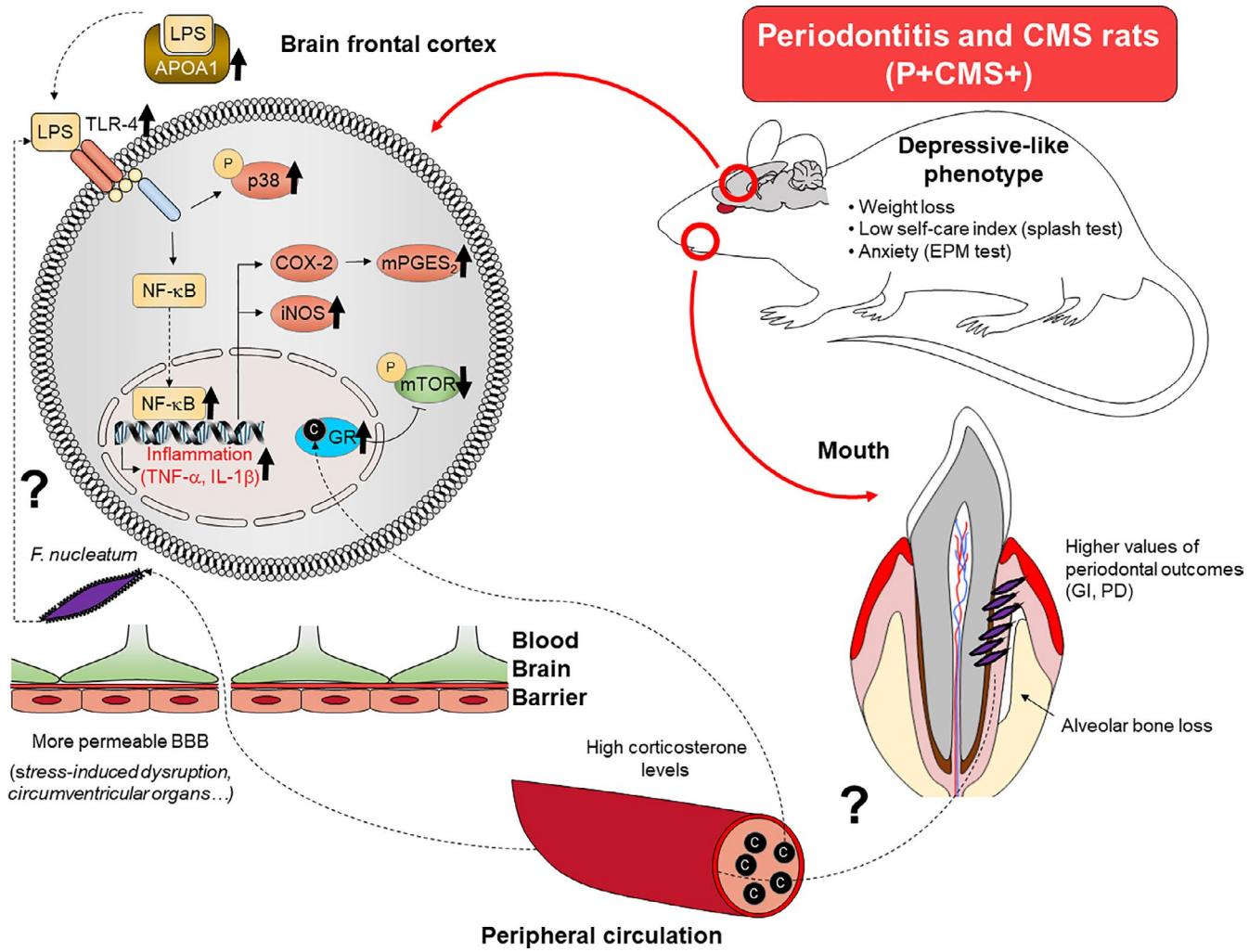

FIGURE 6 Graphical abstract. Combined model of periodontitis and chronic mild stress (CMS). Animals that received the combined protocol (P+CMS+) presented higher values of periodontal outcomes as well as a depressive-like phenotype. At a microbiological level, *Fusobacterium nucleatum* has been found in the brain frontal cortex of P+CMS+ rats, plausibly on account of an increase in blood–brain barrier permeability induced by stress and neuroinflammation. At a molecular level, a decrease in the plasma levels of LPS and LBP was observed in the P+CMS+ group. Apolipoprotein APOA1 protein has been hypothesized as the main candidate for LPS binding and transport to CNS. The binding LPS-TLR-4 triggers a neuroinflammatory response in the frontal cortex driven by NF-kB, iNOS, mPGES and p-p38. High plasma corticosterone and GR levels in the frontal cortex are direct markers of the hypothalamic–pituitary–adrenal (HPA) stress axis dysregulation, and the decline in p-mTOR activation could affect cellular survival in the P+CMS+ group. APOA1, apolipoprotein-A1; BBB, blood–brain barrier; CMS, chronic mild stress; COX-2, cyclooxygenase-2; EPM, elevated plus maze; GI, gingival index; GR, glucocorticoid receptor; IL-1 β, interleukin-1 beta; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; mPGES, microsomal prostaglandin E synthase; m-TOR, mammalian target of rapamycin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; P, periodontitis; PD, probing depth; TLR-4, toll-like receptor 4; TNF-α, tumour necrosis factor-alpha

previously stated as an immunomodulatory condition in its relations to periodontitis (Takada et al., 2004). Our results in the combined model (P+CMS+) are in agreement with previous studies, which found stimulatory effects of stress exposure on the immune response to *P. gingivalis* in mice (Shapira et al., 2000) and to *P. gingivalis* LPS-stimulated secretion of NO in macrophages (Houri-Haddad et al., 2003), respectively. However, in our study, we did not find differences in iNOS expression between the animals exposed to both pathological conditions, compared to the group only submitted to CMS. Although Houri-Haddad et al. (2003) determined nitrite (NO_2) levels (indirect metabolites of NO) and we determine iNOS expression, the differences found could be attributed to differences in the duration and type of the sress (chronic mild vs. acute), in the different protocols used to induce periodontal inflammation, and in the different type of samples (in vivo brain frontal cortex vs. in vitro macrophages).

Another trigger of neuroinflammation, described in previous studies, has been the increased levels of systemic LPS (Pussinen et al., 2007; Martin-Hernandez et al., 2016). However, a decrease in the plasma levels of LPS and LBP, one of its transport proteins, was observed in the present study, in P+CMS+ compared to controls and P+CMS−. This unexpected result requires further studies

using alternative methodologies to corroborate it and elucidate its impact on neuroinflammation. Nevertheless, a plausible hypothesis relies on the potential ability of the combined model to activate a compensatory lipoprotein-mediated transport aimed to regulate the free plasma LPS and its deleterious consequences that could, on the other hand, facilitate its entrance in the CNS (Figure 6).

In this sense, our research group have found evidence supporting the existence of a lipoprotein-mediated transport mechanism, by which peripheral LPS enters the rat brain eliciting TLR-4-dependent neuroinflammation (Vargas-Caraveo et al., 2017). In this vein, the expression of one of the main candidates for LPS binding and transport to CNS, the apolipoprotein APOA1, was explored in the FC of the rats. APOA1 protein expression was increased in P−CMS+ and P+CMS+ compared to both controls and P+CMS−. The consequences of that neuroinflammation are revealed, mainly in the status of the HPA axis. Thus, the stress exposure and the status of the HPA axis (measured by plasma corticosterone and the expression of the GR in brain) emerged also in this experimental setting as a main factor in neuroinflammation. Even though the dysregulation of the stress response is one of the hallmarks in the physiopathology of depression and other psychiatric diseases in humans, being related to alterations in the regulation of innate immunity (García Bueno et al., 2016; Pape et al., 2019), this study has shown an evident additional effect with periodontitis, since P+CMS+ rats showed the highest levels for both corticosterone and GR. Furthermore, stress can induce changes in the mechanisms related to plasticity, survival and cellular death through the regulation of the ubiquitous and multi effector protein kinase m-TOR (Chandran et al., 2013; Guo et al., 2016). m-TOR phosphorylation was downregulated in P+CMS+ rats, possibly due to the described HPA axis hyperactivity. Consequences triggered by this mechanism in our model deserve further research owing to their potential contribution to the FC dysfunction observed in depression, and their involvement in antidepressant responses (Abelaira et al., 2014).

Another mechanism linking periodontitis and depression has been ascribed to the traslocation of periodontal bacteria from the mouth to the brain. Although, under the specific experimental conditions used in the present investigation, a *leaky mouth* process could not be demonstrated, the presence of *F. nucleatum*, in the brain of two rats in the P+CMS+ group, was evidenced, although no differences in periodontal outcomes could be observed in these animals, when compared to others. Previous studies have reported the presence of *P. gingivalis* in the brain of Alzheimer's patients (Dominy et al., 2019), or in animals that have previously received the bacteria (Ilievski et al., 2018). However, this is, in our best knowledge, the first study describing the presence of *F. nucleatum* in brain tissues. Periodontitis induction with *P. gingivalis* not only has led to microbiological changes in the brain, but also to experimental exacerbation of other central nervous system diseases (Polak et al., 2018).

Thus, periodontitis can be related with depression via neuroinflammation by (a) systemic inflammation described in periodontitis patients (Loos, 2005; D'Aiuto et al., 2010) or by (b) direct invasion of periodontal pathogens into the brain (Ilievski et al., 2018). In our study, *F. nucleatum* was only found in the frontal cortex of two animals, while neuroinflammation was a common characteristic among the animals in the P+CMS+ group. Both the systemic inflammation and the translocation of bacteria or bacteria LPS through the blood–brain barrier have been explained before in the literature (Hashioka et al., 2018) and corroborated in this study.

Different limitations have to be acknowledged: (a) the model used to induce periodontitis (oral gavages without a needle) was not able to produce severe ABL, which would have been ideal to amplify the systemic effect (not using a gavage needle or feeding with soft food may have led to a detrimental efficacy of the model); (b) there were difficulties in recording some of the periodontal clinical variables, even when using magnification; (c) the method used to analyse ABL might not be sensitive enough to detect the intrabony component of some osseous defects (Wilensky et al., 2005; de Molon et al., 2018); (d) due to the high intra-group variability of CMS (individual resiliency, non-consanguineous Wistar rats), it was difficult to detect differences between P−CMS+ and P+CMS+ in the behavioural tests; (e) P−CMS− group might have been subjected to a slight stressor derived from the oral gavages administration, but considering its repeated nature and low intensity, it is likely that the HPA stress axis activity is adapted, avoiding persistent physiological alterations that could affect the response to the posterior CMS exposure and (6) there was a tendency for more sucrose intake in the P+ groups compared to P−, what could be considered as a confounding factor in the interpretation of anhedonia (Sidi & Ashley, 1984).

Therefore, and considering the above limitations, it can be concluded that this combined model was adequate to analyse possible mechanisms linking periodontitis and depression reporting a higher inflammatory response both peripheral (plasma and gingival) and central (neuroinflammation). Presence of *F. nucleatum* was identified in the brain tissue of these animals, suggesting a possible mechanism linking periodontitis and depression.

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CONFLICT OF INTEREST

The authors have stated explicitly that they do not have conflicts of interest directly related with this research.

There might be potential indirect sources of conflicts of interests including:

- Personal fees for lecturing for E. Figuero from Colgate, Dentaid, Oral-B and Straumann; for Dr. M. Sanz from Dentaid, Oral-B, Straumann; for D. Herrera from Oral-B, Straumman, Klockner, Dexcel, Dentaid and Colgate.

- Grants (research contracts in university) from Dentaid for M. Sanz, D. Herrera and E. Figuero; from Dentsply and from IMS for M. Sanz; and D. Herrera outside the submitted work.

AUTHOR CONTRIBUTIONS

All authors conceived and planned the experiments. M.M., L.V., D.M.H. and E.M. carried out the experiments with the animals. M.J.M. and N.A. carried out the microbiological analysis. D.M.H. and K.M. carried out the immunological analyses. E.F., D.M.H and B.G.B. performed the statistical analyses. B.G.B., E.F., J.C.L, M.S. and D. H. contributed to the interpretation of the results. M.M., D.M.H, B.G.B and E.F. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

ETHICAL STATEMENT

This preclinical in vivo study was carried out following European (2010/63/UE) and Spanish (RD53/2013) legislation. The protocol was approved by the regional authorities (PROEX 087/18) and the Ethical Committee of Animal Experimentation at the Complutense University of Madrid, where the study was carried out in the Experimental Animal Center following the three Rs principles of care (Replacement, Reduction and Refinement).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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APPENDIX 1

LEGISLATION AND COMMITTEE FOLLOWED DURING THE STUDY

This preclinical in vivo study was carried out following European (2010/63/UE) and Spanish (RD53/2013) legislation. The protocol was approved by the regional authorities (PROEX 087/18) and the Ethical Committee of Animal Experimentation at the Complutense University of Madrid, where the study was carried out in the Experimental Animal Center following the three Rs principles of care (Replacement, Reduction and Refinement).

APPENDIX 2

CONDITIONS FOR ANIMALS PRIOR TO EXPERIMENTS

Male Wistar Hannover rats (HsdRccHan:Wist, Envigo, Spain) (230–250 g) were randomly distributed in open polycarbonate cages into four study groups according to the different interventions that would be performed, and maintained at a constant temperature of 24 ± 2°C and a relative humidity of 70 ± 5% in a 12 h light–dark cycle (lights on at 8:00 AM). Animals were fed with standard pellet chow (A04 SAFE, Scientific Animal Food and Engineering©, Augy, France) with free access to fresh tap water and were maintained under constant conditions for 7 days prior to experiments.

APPENDIX 3

PRIMER-PROBE SEQUENCES AND CONDITIONS FOR QPCR

Bacterial species	Sequence (5′–3′
Porphyromonas gingivalis	F: GCGCTCAACGTTCAGC R: CACGAATTCCGCCTGC P: 6FAM-CACTGAACTCAAGCCCGGCAGTTTCAA-TAMRA
Fusobacterium nucleatum	F: GGATTTATTGGGCGTAAAGC R: GGCATTCCTACAAATATCTACGAA P: 6FAM-CTCTACACTTGTAGTTCCG-TAMRA

Abbreviations: F, forward; P, probe; R, reverse.

Quantitative polymerase chain reaction (qPCR) conditions

PCR amplification was performed in a solution containing 2× TaqMan of master mixture (LC480 Probes Master, Roche Diagnostic GmbH, Mannheim, Germany), optimal primers and probes concentrations (300, 300 and 300 nmol/L for *P. gingivalis*; 600, 600 and 300 nM for *F. nucleatum*) and DNA from brain and blood samples. The samples were submitted to amplification cycles at 95°C for 15 s and 60° for 1 min. All assays were developed with a linear quantitative detection range established by the slope range of 3.3–3.6 cycles/log decade, r2 > 0.997 and an efficiency range of 1.9–2.0. The quantification was determined from the standard curves from 10^1 to 10^9 of purified genomic DNA of *P. gingivalis* and *F. nucleatum*.

APPENDIX 4

EVALUATION OF DEPRESSION-LIKE BEHAVIOUR

Elevated plus maze (EPM) measures anxiety in rats (Pellow et al., 1985). EPM comprised four wooden arms (50 × 10 cm), two open and two enclosed (40 cm high walls) perpendicularly organized. The maze was elevated 50 cm above the floor, and the room was suitably illuminated. The test consisted of placing each animal in the centre of the EPM, facing an open arm, for a 5-min recording session. A trained researcher (DMH) performed a blind scoring of the total time spent in the open arms and the number of entrances per arm.

Splash test (ST) assesses the grooming behaviour, linked to anhedonia and self-caring, subsequently to CMS exposure for 5 min after spraying a 10% sucrose solution on the dorsal coat of the rats under red light conditions during their activity phase (Yalcin et al., 2005). The test was recorded using a video camera. Grooming and latency times (time elapsed before rats started the grooming behaviour) were analysed by a trained researcher blinded to the experimental conditions.

A decrease in sucrose preference has been proposed as an anhedonia-like feature after exposure to an experimental model of depression (Grippo et al., 2005). Food and water from each cage were removed 12 h before the test. Two pre-weighed bottles containing water and 1% sucrose were then placed on the cages (one animal per cage), and animals had free access to them for a period of 1 h. After that, the bottles were removed and weighed. Differences between weights represent each fluid intake (g), and the sucrose per total fluid ratio indicates the sucrose preference. To avoid a side bias, the positions of the two bottles alternate every week.

APPENDIX 5

STUDIED BIOMARKERS

Mediator	Role in neuroinflammation	Analysis technique
IL-1β	Pro-inflammatory cytokine	RT-PCR
TNF-α	Pro-inflammatory cytokine	RT-PCR
TLR-4	Innate immune receptor for LPS	RT-PCR/WB
NF-κB	Nuclear transcription factor controlling inflammatory response	RT-PCR/WB
COX-2	Prostanoid formation enzyme	RT-PCR
iNOS	Inducible enzyme involved in the production of NO	RT-PCR/WB
mPGES	Enzyme involved in the synthesis of PGE.	RT-PCR
p38	MAPK related to inflammation	WB
LPS	Component of the outer membrane of Gram – bacteria	LAL assay
LBP	LPS binding protein	ELISA
APO A1	Major component of HDL, candidate for LPS transport to the CNS	WB
Corticosterone	Predominant glucocorticoid in rodents, involved in stress and immune reactions	ELISA
Glucocorticoid receptor	Bind to glucocorticoids and activates transcription of stress-responsive genes	WB
m-TOR	Protein kinase regulating several processes such as cell survival and stress responses	WB

Abbreviations: APO-A1, apolipoprotein-A1; COX, cyclooxygenase; ELISA, enzyme-linked immunosorbent assay; GR, glucocorticoid receptor; IL-1 β, interleukin-1 beta; iNOS, inducible nitric oxide synthase; LAL, limulus amebocyte lysate; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; mPGES, microsomal prostaglandin E synthase; m-TOR, mechanistic target of rapamycin kinase; NF- κB, nuclear factor kappa-light-chain-enhancer of activated B cells; p38, phospho-p38 mitogen activated protein kinase; RT-PCR, reverse transcriptase polymerase chain reaction; TLR, toll like receptor; TNF- α, tumour necrosis factor-alpha; WB, western blot.

APPENDIX 6

NUCLEAR EXTRACTION AND TOTAL HOMOGENATE PROCEDURES

Briefly, the tissue (frontal cortex, FC) was homogenized in 300 mL buffer (10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (pH 7.9); 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L egtazic acid (EGTA), 10 mmol/L KCl, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.1 mg m/L aprotinin, 1 mg/mL leupeptin, 1 mg/mL Na-p-tosyll-lysine chloromethylketone, 5 mmol/L NaF, 1 mmol/L $NaVO_4$, 0.5 mol/L sucrose, and 10 mmol/L Na_2MoO_4). After 15 min, Nonidet P-40® (Roche, Mannheim, Germany) was added to reach a 0.5% concentration. The tubes were gently vortexed for 15 s, and nuclei were collected by centrifugation at 5000 *g* for 5 min. The pellets were resuspended in 100 μl buffer supplemented with 20% glycerol and 0.4 mol/L KCl and gently shaken for 30 min at 4°C. Nuclear protein extracts were obtained by centrifugation at 13,000 *g* for 5 min, and aliquots of the supernatant were stored at −80°C. All steps of the fractionation were carried out at 4°C.

For FC total homogenates, FC tissue were homogenized in PBS1x with protease inhibitor cocktail (cOmplete, Roche). After centrifugation at 19,083 *g* for 10 min, supernatant was collected as the total homogenate. All steps were carried out at 4°C.

APPENDIX 7

WESTERN BLOT PROCEDURES

After adjusting protein levels in the homogenates and mixing them with Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) 20 ml (1 mg/ml), they were loaded and the proteins size-separated in 8% SDS-polyacrylamide gel electrophoresis (90 V). After the gel electrophoresis, the membranes were blocked in 30 ml Tris-buffered saline containing 0.1% Tween 20 and 5% bobbin serum albumin (BSA). Then the membranes were incubated with specific primary antibodies (GR (sc-1004, Santa Cruz Biotechnology, 1:1000); p65 (sc-372, Santa Cruz Biotechnology, 1:1000); TLR-4 (sc-16240, Santa Cruz Biotechnology, 1:1000); iNOS (sc-650, Santa Cruz Biotechnology, 1:750, 2% BSA); p-p38 (sc-17852R, Santa Cruz Biotechnology, 1:1000); p38α/β (sc-7972, Santa Cruz Biotechnology, 1:1000); APO A1 (ab-20453, Abcam, 1:1000); p-mTOR (#5536, Cell Signaling, 1:1000); m-TOR (2983, Cell Signaling, 1:1000). After washing with a TBS-Tween solution the membranes were incubated with the respective horseradish peroxidase-conjugated secondary antibodies for 90 min at room temperature and revealed by ECL™-kit following manufacturer's instructions (Amersham Ibérica, RTN2236; Spain).

Blots were imaged using an Odyssey® Fc System (Li-COR Biosciences) and quantified by densitometry (NIH ImageJ® software). All densitometries are expressed in arbitrary units of optical density (OD). Several exposition times were analysed to ensure linearity of the band intensities. Loading controls (blots shown in the respective figures) were GAPDH (Sigma G8795) for the nuclear extracts, and β-actin (Sigma A5441) for the total homogenates.

APPENDIX 8

PRIMER SEQUENCES AND CONDITIONS FOR RT-PCR

Protein	*Forward*	*Reverse*
IL-1β	ACCTGCTAGTGTGTGATGTTCCCA	AGGTGGAGAGCTTTCAGCTCACAT
TNF-α	CTGGCCAATGGCATGGATCTCAAA	ATGAAATGGCAAATCGGCTGACGG
TLR-4	ACATCAGAGGAAGAACAAGAAGCA	CGGAAATTGTAAACATAATGGGTTT
COX-2	CTTCGGGAGCACAACAGAG	GCGGATGCCAGTGATAGAG
iNOS	GGACCACCTCTATCAGGAA	CCTCATGATAACGTTTCTGGC
NF-κB	CATGCGTTTCCGTTACAAGTGCGA	TGGGTGCGTCTTAGTGGTATCTGT
mPGES	GGTGAAGCAAATGTTCCCAGCTCA	TTTAGCGGTTGGTCAAAGCCCATC
β-tubulin	CCCTCGCCATGGTAAATACAT	ACTGGATGGTACGCTTGGTCT

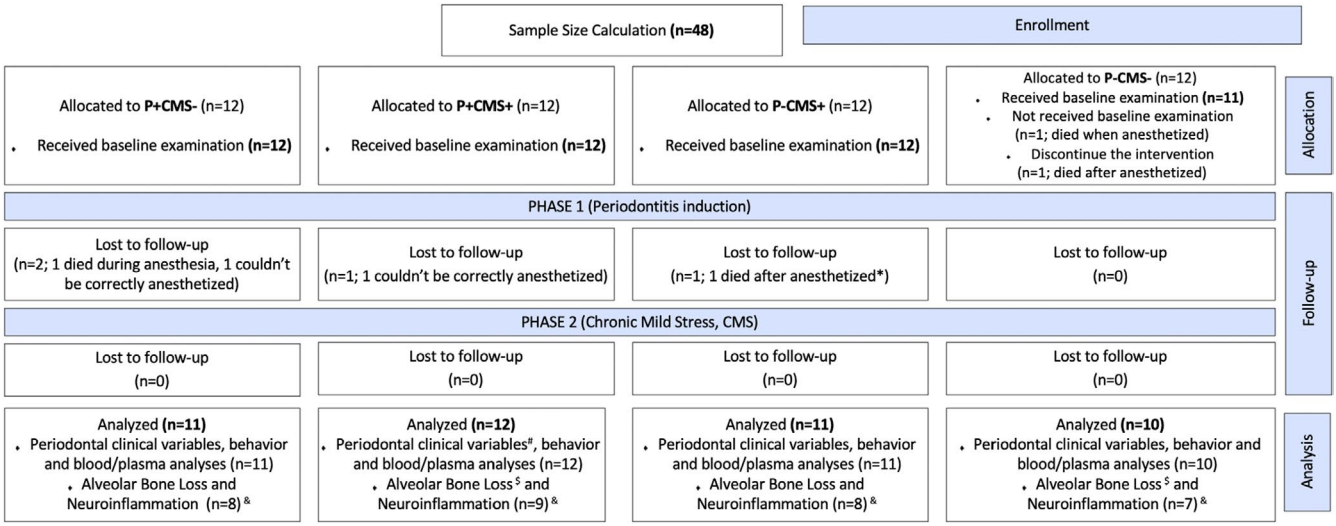
Abbreviations: COX-2, cyclooxygenase-2; IL-1 β, interleukin-1 beta; iNOS, inducible nitric oxid synthase; mPGES, microsomal prostaglandin E synthase; NF- κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TLR-4, toll like receptor-4; TNF- α, tumour necrosis factor-alpha.

Reverse transcriptase (RT) PCR conditions

35–40 cycles of denaturation at 95°C for 10 s, annealing at 58–65°C for 15 s depending on the specific set of primers, and extension at 72°C for 20 s. Reactions were carried out in presence of SYBR green (Quantimix Easy Master Mix Biotools, B&M labs 10607-4154) in a 20 μl reaction in a Rotor-Gene (Corbett Research, Mortlake, NSW, Australia). Relative mRNA concentrations were calculated from the take-off point of reactions using included software, and β-tubulin levels used as housekeeper.

APPENDIX 9

FLOW DIAGRAM



*For animals that died after anesthetized, periodontal clinical variables were registered.

#1 animal had problems with anaesthesia after phase 2, and only some periodontal clinical variables could be registered.

$Whenever an anatomical references was not clear, the measurement of alveolar bone loss was not analysed.

&Three animals were perfused for further analysis (data not shown). In these animals, alveolar bone loss, inflammatory mediators in frontal cortex and microbiological outcomes could not be registered.

APPENDIX 10

EVALUATION OF DEPRESSIVE-LIKE BEHAVIOUR

						Post- hoc comparison				
							95% CI			
		n	Mean	SD	Global *p* value (ANOVA)	Specific comparison	Mean Difference	Lower Bound	Upper Bound	Post-hoc *p* –value (ANOVA)
Weight gain (%) DAY 1	P+CMS−	11	100.00	6.60	.858	P+CMS− vs. P+CMS+				>.999
	P+CMS+	12	100.00	6.31		P+CMS− vs. P−CMS+				>.999
	P−CMS+	11	100.00	8.15		P−CMS− vs. P+CMS−				>.999
	P−CMS−	10	100.00	10.15		P−CMS+ vs. P+CMS+				>.999
						P−CMS− vs. P+CMS+				>.999
						P−CMS− vs. P−CMS+				>.999
Weight gain (%) DAY 7	P+CMS−	11	101.00	1.27	**<.001**	P+CMS− vs. P+CMS+	3.19	1.83	4.50	**<.001**
	P+CMS+	12	97.60	1.13		P+CMS− vs. P−CMS+	3.69	2.30	5.10	**<.001**
	P−CMS+	11	97.10	1.35		P−CMS− vs. P+CMS−	2.62	1.20	4.03	**<.001**
	P−CMS−	10	103.00	0.85		P−CMS+ vs. P+CMS+	−0.50	−1.85	0.85	.896
						P−CMS− vs. P+CMS+	5.81	4.40	7.20	**<.001**
						P−CMS− vs. P−CMS+	6.31	4.89	7.70	**<.001**
Weight gain (%) DAY 14	P+CMS−	11	103.40	1.04	**<.001**	P+CMS− vs. P+CMS+	5.79	4.13	7.45	**<.001**
	P+CMS+	12	97.60	1.62		P+CMS− vs. P−CMS+	5.83	3.60	8.06	**<.001**
	P−CMS+	11	97.58	2.20		P−CMS− vs. P+CMS−	1.86	0.02	3.70	**.047**
	P−CMS−	10	105.00	1.64		P−CMS+ vs. P+CMS+	−0.04	−2.43	2.30	>.999
						P−CMS− vs. P+CMS+	7.65	5.60	9.70	**<.001**
						P−CMS− vs. P−CMS+	7.69	5.21	10.20	**<.001**
Weight gain (%) DAY 21	P+CMS−	11	106.00	1.06	**<.001**	P+CMS− vs. P+CMS+	5.86	4.02	7.70	**<.001**
	P+CMS+	12	100.10	1.85		P+CMS− vs. P−CMS+	6.32	4.30	8.30	**<.001**
	P−CMS+	11	99.60	1.94		P−CMS− vs. P+CMS−	−0.39	−2.70	1.90	.990
	P−CMS−	10	105.60	2.16		P−CMS+ vs. P+CMS+	−0.45	−2.76	1.85	.990
						P−CMS− vs. P+CMS+	5.48	2.90	8.03	**<.001**
						P−CMS− vs. P−CMS+	5.90	3.20	8.50	**<.001**
SUCROSE PREFERENCE TEST	P+CMS−	10	75.00	13.00	.183	P+CMS− vs. P+CMS+				.961
Sucrose preference (%)	P+CMS+	12	77.00	8.00		P+CMS− vs. P−CMS+				.179
	P−CMS+	10	67.00	9.00		P−CMS− vs. P+CMS−				.596
	P−CMS-	10	72.92	15.00		P−CMS+ vs. P+CMS+				.347
						P−CMS− vs. P+CMS+				.837
						P−CMS− vs. P−CMS+				.869
SUCROSE PREFERENCE TEST	P+CMS−	10	11.90	3.50	.242	P+CMS− vs. P+CMS+				.984
Sucrose intake	P+CMS+	12	11.15	3.10		P+CMS− vs. P−CMS+				.408
	P−CMS+	10	8.50	3.36		P−CMS− vs. P+CMS−				.968
	P−CMS−	9	9.80	4.60		P−CMS+ vs. P+CMS+				.209

						Post- hoc comparison				
							95% CI			
		n	Mean	SD	Global *p* value (ANOVA)	Specific comparison	Mean Difference	Lower Bound	Upper Bound	Post-hoc *p* –value (ANOVA)
						P−CMS− vs. P+CMS+				.841
						P−CMS− vs. P−CMS+				.683
SPLASH TEST	P+CMS−	10	1.84	0.20	**.006**	P+CMS− vs. P+CMS+	0.34	−0.05	0.73	.110
log10 Grooming time	P+CMS+	12	1.50	0.40		P+CMS− vs. P−CMS+	0.48	0.04	0.91	**.028**
	P−CMS+	10	1.36	0.40		P−CMS− vs. P+CMS−	−0.05	−0.35	0.26	.990
	P−CMS−	8	1.79	0.22		P−CMS+ vs. P+CMS+	−0.14	−0.63	0.36	.960
						P−CMS− vs. P+CMS+	0.29	−0.11	0.70	.260
						P−CMS− vs. P−CMS+	0.43	−0.02	0.87	.064
EPM test	P+CMS−	10	194.00	27.00	**.002**	P+CMS− vs. P+CMS+	55.10	18.70	91.40	**.001**
Time in open arms	P+CMS+	12	139.00	37.50		P+CMS− vs. P−CMS+	39.65	2.60	76.70	**.032**
	P−CMS+	11	154.50	27.70		P−CMS− vs. P+CMS−	−25.21	−64.21	13.80	.320
	P−CMS−	9	169.00	32.00		P−CMS+ vs. P+CMS+	15.45	−20.00	51.00	.650
						P−CMS− vs. P+CMS+	29.90	7.54	67.30	.150
						P−CMS− vs. P−CMS+	14.43	−23.70	52.60	.740

Abbreviations: CI, confidence interval; CMS, chronic mild stress; EPM, elevated plus maze; P, periodontitis; SD, standard deviation. Bold values indicate p values ≤ 0.05.

APPENDIX 11

QUANTIFICATION OF INFLAMMATORY MEDIATORS IN FRONTAL CORTEX BRAIN SAMPLES

						Post- hoc comparison				
							95% CI			
		n	Mean	SD	Global *p* value (ANOVA)	Specific comparison	Mean Difference	Lower Bound	Upper Bound	Post-hoc *p*-value (ANOVA)
TNF- α mRNA	P+CMS−	8	151.80	34.77	**<.001**	P+CMS− vs. P+CMS+	−85.73	−187.70	16.24	.116
	P+CMS+	8	237.50	80.15		P+CMS− vs. P−CMS+	−40.11	−101.00	20.74	.321
	P−CMS+	8	191.90	43.68		P−CMS− vs. P+CMS−	−51.80	−96.00	−7.60	**.020**
	P−CMS−	6	100.00	11.48		P−CMS+ vs. P+CMS+	−45.62	−149.00	58.00	.708
						P−CMS− vs. P+CMS+	−137.50	−239.60	−35.44	**.010**
						P−CMS− vs. P−CMS+	−91.92	−147.30	−36.52	**.002**
IL-1 β mRNA	P+CMS−	8	136.30	42.62	**<.001**	P+CMS− vs. P+CMS+	−92.00	−154.00	−30.00	**.002**
	P+CMS+	8	228.30	59.52		P+CMS− vs. P−CMS+	−63.68	−126.00	−1.70	**.040**
	P−CMS+	8	200.00	41.15		P−CMS− vs. P+CMS−	−36.32	−100.50	27.90	.420
	P−CMS−	7	100.00	31.75		P−CMS+ vs. P+CMS+	−28.32	−90.30	33.70	.600
						P−CMS− vs. P+CMS+	−128.30	−192.50	−64.16	**<.001**
						P−CMS− vs. P−CMS+	−100.00	−164.20	−35.80	**.001**
TLR-4 mRNA	P+CMS−	8	109.00	7.33	**.036**	P+CMS− vs. P+CMS+	−6.68	−24.13	10.76	.722
	P+CMS+	8	115.70	13.43		P+CMS− vs. P−CMS+	−10.20	−27.65	7.24	.395
	P−CMS+	8	119.20	11.85		P−CMS− vs. P+CMS−	−9.01	−27.07	9.04	.530
	P−CMS−	7	100.00	17.17		P−CMS+ vs. P+CMS+	3.52	−13.93	20.97	.945
						P−CMS− vs. P+CMS+	−15.70	−33.76	2.36	.105

						Post- hoc comparison				
							95% CI			
		n	Mean	SD	Global *p* value (ANOVA)	Specific comparison	Mean Difference	Lower Bound	Upper Bound	Post-hoc *p*-value (ANOVA)
						P−CMS− vs. P−CMS+	−19.22	−37.28	−1.15	**.033**
TLR-4 protein	P+CMS−	8	141.80	40.35	**.009**	P+CMS− vs. P+CMS+	−45.96	−118.00	26.10	.320
	P+CMS+	9	187.80	6214		P+CMS− vs. P−CMS+	−46.54	−120.70	27.60	.330
	P−CMS+	8	188.40	68.78		P−CMS− vs. P+CMS−	−41.82	−118.60	34.92	.458
	P−CMS−	7	100.00	34.6		P−CMS+ vs. P+CMS+	0.58	−71.50	72.60	>.999
						P−CMS− vs. P+CMS+	−87.78	−162.50	−13.06	**.016**
						P−CMS− vs. P−CMS+	−88.36	−165.10	−11.62	**.019**
NF-kB mRNA	P+CMS−	8	117.20	15.98	**.011**	P+CMS− vs. P+CMS+	−8.20	−28.49	12.10	.756
	P+CMS+	7	125.40	5.86		P+CMS− vs. P−CMS+	−7.79	−36.15	20.60	.959
	P−CMS+	8	124.40	20.49		P−CMS− vs. P+CMS−	−17.16	−37.50	3.18	.107
	P−CMS−	6	100.00	2.49		P−CMS+ vs. P+CMS+	−0.41	−26.40	25.60	>.999
						P−CMS− vs. P+CMS+	−25.36	−33.70	−17.02	**<.001**
						P−CMS− vs. P−CMS+	−24.95	−51.10	1.19	.062
NF-kB nuclear protein	P+CMS−	7	82.66	22.74	**.007**	P+CMS− vs. P+CMS+	−67.83	−137.40	1.74	**.057**
	P+CMS+	9	150.50	59.66		P+CMS− vs. P−CMS+	−24.76	−59.50	9.97	.241
	P−CMS+	8	107.40	19.7		P−CMS− vs. P+CMS−	17.34	−21.90	56.60	.718
	P−CMS−	7	100.00	23.97		P−CMS+ vs. P+CMS+	−43.06	−112.00	25.86	.346
						P−CMS− vs. P+CMS+	−50.48	−120.30	19.32	.220
						P−CMS− vs. P−CMS+	−7.42	−43.50	28.66	.989
iNOS mRNA	P+CMS−	8	129.40	57.31	**.022**	P+CMS− vs. P+CMS+	−72.23	−192.00	47.53	.401
	P+CMS+	8	201.60	90.94		P+CMS− vs. P−CMS+	−34.92	−111.00	41.00	.680
	P−CMS+	8	164.30	37.44		P−CMS− vs. P+CMS−	−29.38	102.10	43.40	.730
	P−CMS−	6	100.00	12.31		P−CMS+ vs. P+CMS+	−37.31	153.00	78.20	.890
						P−CMS− vs. P+CMS+	−101.60	−217.50	14.30	.091
						P−CMS− vs. P−CMS+	−64.30	−112.00	−16.70	**.008**
iNOS protein	P+CMS−	7	114.60	20.16	**<.001**	P+CMS− vs. P+CMS+	−47.53	−101.00	5.80	.090
	P+CMS+	9	162.20	45.16		P+CMS− vs. P−CMS+	−43.90	−75.20	−12.60	**.005**
	P−CMS+	8	158.20	18.36		P−CMS− vs. P+CMS−	−14.65	−43.85	14.50	.570
	P−CMS−	7	100.00	12.35		P−CMS+ vs. P+CMS+	−3.63	−56.20	49.00	>.999
						P−CMS− vs. P+CMS+	−62.18	−114.30	−10.10	**.018**
						P−CMS− vs. P−CMS+	−58.54	−83.55	−33.54	**<.001**
mPGES mRNA	P+CMS−	8	148.50	30.89	**.001**	P+CMS− vs. P+CMS+	−7.98	−44.11	28.15	.929
	P+CMS+	8	156.40	27.35		P+CMS− vs. P−CMS+	12.46	−23.67	48.60	.781
	P−CMS+	8	136.00	23.29		P−CMS− vs. P+CMS−	−48.46	−85.86	−11.06	**.007**
	P−CMS−	7	100.00	22.79		P−CMS+ vs. P+CMS+	−20.45	−56.58	15.68	.423
						P−CMS− vs. P+CMS+	−56.44	−93.84	−19.04	**.001**
						P−CMS− vs. P−CMS+	−35.99	−73.39	1.41	.062

						Post- hoc comparison				
							95% CI			
		n	Mean	SD	Global *p* value (ANOVA)	Specific comparison	Mean Difference	Lower Bound	Upper Bound	Post-hoc *p*-value (ANOVA)
log10 p-p38/ p38 α/ β ratio protein	P+CMS−	8	2.03	0.14	**.002**	P+CMS− vs. P+CMS+	−0.16	−0.30	−0.02	**.020**
	P+CMS+	9	2.18	0.031		P+CMS− vs. P−CMS+	−0.13	−0.28	0.02	.100
	P−CMS+	7	2.16	0.13		P−CMS− vs. P+CMS−	−0.03	−0.18	0.12	.930
	P−CMS−	7	2.00	0.09		P−CMS+ vs. P+CMS+	−0.03	−0.17	0.12	.950
						P−CMS− vs. P+CMS+	−0.19	−0.34	0.04	**.007**
						P−CMS− vs. P−CMS+	−0.17	−0.32	−0.01	**.035**

Abbreviations: CI, confidence interval; CMS, chronic mild stress; COX, cyclooxygenase; IL-1 β, interleukin-1 beta; iNOS, inducible nitric oxid synthase; mPGES, microsomal prostaglandin E synthase; NF- κB, nuclear factor kappa-light-chain-enhancer of activated B cells; P, periodontitis; SD, standard deviation; TLR, toll like receptor; TNF- α, tumour necrosis factor-alpha. Bold values indicate p values ≤ 0.05.

APPENDIX 12

POSSIBLE ORIGIN AND CONSEQUENCES OF NEUROINFLAMMATION

						Post- hoc comparison				
							95% CI			
		n	Mean	SD	Global *p* value (ANOVA)	Specific comparison	Mean difference	Lower bound	Upper bound	Post-hoc *p*-value (ANOVA)
LPS plasma levels	P+CMS−	11	0.69	0.20	**.012**	P+CMS− vs. P+CMS+	0.26	0.01	0.51	**.039**
	P+CMS+	12	0.43	0.16		P+CMS− vs. P−CMS+	0.17	−0.09	0.43	.310
	P−CMS+	10	0.52	0.26		P−CMS− vs. P+CMS−	0.02	−0.23	0.28	.990
	P−CMS−	10	0.71	0.26		P−CMS+ vs. P+CMS+	0.09	−0.16	0.34	.780
						P−CMS− vs. P+CMS+	0.28	0.03	0.54	**.026**
						P−CMS− vs. P−CMS+	0.19	−0.07	0.46	.230
log10 LBP plasma levels	P+CMS−	11	1.82	0.14	**<.001**	P+CMS− vs. P+CMS+	0.30	0.14	0.46	**<.001**
	P+CMS+	12	1.55	0.21		P+CMS− vs. P−CMS+	0.11	−0.06	0.29	.305
	P−CMS+	10	1.74	0.13		P−CMS− vs. P+CMS−	0.05	−0.13	0.22	.890
	P−CMS−	10	1.87	0.12		P−CMS+ vs. P+CMS+	0.19	0.02	0.35	**.021**
						P−CMS− vs. P+CMS+	0.35	0.18	0.52	**<.001**
						P−CMS− vs. P−CMS+	0.16	−0.02	0.34	.087
APOA1 protein	P+CMS−	8	109.60	23.00	**<.001**	P+CMS− vs. P+CMS+	−50.73	−91.91	−9.56	**.011**
	P+CMS+	9	160.30	13.92		P+CMS− vs. P−CMS+	−50.11	−92.50	−7.70	**.016**
	P−CMS+	8	159.70	53.45		P−CMS− vs. P+CMS−	−9.61	−53.50	34.20	.930
	P−CMS−	7	100.00	16.97		P−CMS+ vs. P+CMS+	−0.62	−41.80	40.55	>.999
						P−CMS− vs. P+CMS+	−60.35	−103.10	17.60	**.003**
						P−CMS− vs. P−CMS+	−59.72	−103.60	−15.90	**.004**
Corticosterone plasma levels	P+CMS−	11	210.00	93.00	**.040**	P+CMS− vs. P+CMS+	−55.98	−142.40	30.40	.318
	P+CMS+	12	266.00	91.00		P+CMS− vs. P−CMS+	9.13	−81.30	99.50	.990

						Post- hoc comparison				
							95% CI			
		n	Mean	SD	Global *p* value (ANOVA)	Specific comparison	Mean difference	Lower bound	Upper bound	Post-hoc *p*-value (ANOVA)
	P−CMS+	10	201.00	50.00		P−CMS− vs. P+CMS−	−40.38	−131.00	50.00	.630
	P−CMS−	10	170.00	58.00		P−CMS+ vs. P+CMS+	−65.12	−153.70	23.50	.216
						P−CMS− vs. P+CMS+	−96.37	−185.00	−7.70	**.028**
						P−CMS− vs. P−CMS+	−31.25	−124.00	61.30	.800
GR nuclear protein	P+CMS−	7	80.00	23.00	**.006**	P+CMS− vs. P+CMS+	−80.30	−141.40	−19.20	**.006**
	P+CMS+	9	161.00	67.00		P+CMS− vs. P−CMS+	−19.77	−82.52	42.98	.824
	P−CMS+	8	100.10	20.20		P−CMS− vs. P+CMS−	19.63	−45.17	84.44	.840
	P−CMS−	7	100.00	41.00		P−CMS+ vs. P+CMS+	−60.53	−119.40	−1.62	**.042**
						P−CMS− vs. P+CMS+	−60.67	−121.80	0.43	.052
						P−CMS− vs. P−CMS+	−0.14	−62.88	62.61	>.999
p-mTOR/ m-TOR ratio protein	P+CMS−	8	127.60	27.00	**.003**	P+CMS− vs. P+CMS+	55.03	19.23	91.00	**.001**
	P+CMS+	8	72.60	13.00		P+CMS− vs. P−CMS+	29.50	−6.25	65.35	.130
	P−CMS+	8	98.00	29.00		P−CMS− vs. P+CMS−	−27.60	−65.00	9.45	.200
	P−CMS−	7	100.00	31.00		P−CMS+ vs. P+CMS+	25.50	−10.32	61.30	.230
						P−CMS− vs. P+CMS+	27.40	−9.64	64.50	.200
						P−CMS− vs. P−CMS+	1.93	−35.12	39.00	.990

Abbreviations: APO-A1, apolipoprotein-A1; CI, confidence interval; CMS, chronic mild stress; EU, endotoxin units; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GR, glucocorticoid receptor; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; m-TOR, mechanistic target of rapamycin kinase; P, periodontitis; SD, standard deviation. Bold values indicate p values ≤ 0.05.