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Strong and persistent microbial and inflammatory stimuli overcome the genetic predisposition to higher matrix metalloproteinase-1 (MMP-1) expression: a mechanistic explanation for the lack of association of *MMP1-1607* single-nucleotide polymorphism genotypes with MMP-1 expression in chronic periodontitis lesions

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Abstract

Aims: Our objective was to evaluate the association between the *MMP1-1607* single-nucleotide polymorphism (SNP), periodontopathogens and inflammatory cytokines with matrix metalloproteinase-1 (MMP-1) mRNA levels in vitro and in vivo.

Materials and Methods: This study investigated the influence of genetic (*MMP1-1607* SNP), microbial (*Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Actinobacillus actinomycetemcomitans*) and inflammatory [tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)] factors on the determination of MMP-1 mRNA levels in periodontal tissues of non-smoker chronic periodontitis (CP, $N = 178$) and control (C, $N = 190$) groups. The effects of single and repeated lipopolysaccharide (LPS) and inflammatory cytokine stimulation of macrophages with distinct *MMP1-1607* SNP genotypes were also investigated.

Results: In healthy tissues, the *MMP1-1607* 2G allele was associated with higher MMP-1 levels while in CP MMP-1 levels were associated with the presence and load of periodontopathogens, and also with TNF-α and IL-1β expression irrespective of the *MMP1-1607* genotype. In vitro data demonstrate that in 2G macrophages low- and intermediate-dose LPS and TNF-α+IL-1β stimulation was associated with increased MMP-1 expression, while strong and repeated stimulation resulted in higher MMP-1 levels irrespective of the *MMP1-1607* genotype.
Conclusion: Our data demonstrate a limited role for *MMP1-1607* SNP in periodontitis, where the extensive chronic antigenic challenge exposure overcomes the genetic control and plays a major role in the determination of MMP-1 expression.

Key words: cytokines; MMP-1; periodontal disease; periodontopathogens; single-nucleotide polymorphism

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Conflict of interest and source of funding statement

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Periodontal diseases are chronic infectious inflammatory diseases characterized by the extensive destruction of the matrix of soft and mineralized attachment tissues of the teeth, being the most prevalent form of bone pathology in humans and a modifying factor of the systemic health of patients (Mombelli 2003, Tonetti & Claffey 2005). The bacterial biofilm attached to the tooth surface hosts a broad diversity of Gram-negative species, including some typical periodontopathogens, such as *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* (which comprised the red complex) and *Actinobacillus actinomycetemcomitans* (Ximénez-Fyvie et al. 2000, Feng & Weinberg 2006). While the red complex is strongly associated with chronic periodontitis, *A. actinomycetemcomitans* is considered to be the main aetiological agent of aggressive periodontitis and of a significant nuperiodontitis (Ximénez-Fyvie et al. 2000, Feng & Weinberg 2006). The chronic host response raised against these periodontopathogens involves an intense and persistent expression of several pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β; Graves & Cochran 2003), which induce a protease-mediated catabolic response leading to the irreversible periodontal tissue attachment loss (Nishikawa et al. 2002, Garlet et al. 2004).

Among host proteases active in the periodontal environment, matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent proteases, play a role in the degradation and remodelling of both extracellular and bone matrix proteins (Reynolds et al. 1994, Hannas et al. 2007). MMP-1 is the main proteolytic enzyme with the capacity to cleave type I and III collagen fibres, which are the most abundant components of the periodontal tissue matrix (Verstappen & Von den Hoff 2006, Hannas et al. 2007). MMP-1 levels are relatively low in healthy periodontal tissues, which are thought to contribute to its physiological turnover (Reynolds et al. 1994, van der Zee et al. 1997). However, dysregulation of MMP transcription and activation can lead to pathological processes, including periodontal diseases (Reynolds et al. 1994, Hannas et al. 2007). In fact, MMPs play active roles in the destructive processes throughout periodontal disease, and MMP inhibitors are used as adjunct therapy to human periodontitis (Verstappen & Von den Hoff 2006). Interestingly, studies demonstrate a high individual variation in the levels of MMP-1 in diseased periodontium, which is thought to influence the disease outcome considerably (Garlet et al. 2004, Verstappen & Von den Hoff 2006, Zhou & Windsor 2007). However, the factors underlying the determination of MMP-1 levels in periodontal tissues remain unknown.

Polymorphic loci were identified in the promoter region of the *MMP1* gene, and are thought to account for heritable differences in MMP-1 expression (Kanamori et al. 1999, Ye 2000, Sternlicht & Werb 2001). A G (guanine) addition at the −1607 position of the *MMP1* gene promoter (*MMP1-1607*, rs: 1799750) was particularly associated with increased transcriptional activity, characterizing therefore a functional polymorphism that has been associated with different diseases (Kanamori et al. 1999, Ye 2000, Pirhan et al. 2008). In recent studies, both *MMP1-519* and *MMP1-1607* single-nucleotide polymorphisms (SNPs) were described to be associated with the severity of chronic periodontitis in a Turkish population by one research group (Pirhan et al. 2008), while another group obtained an opposite result (Ustun et al. 2008). Indeed, the relatively small sample investigated regarding the putative functionality of the SNPs, in addition to the non-observance of potential factors involved in the regulation of MMP-1 levels (i.e. smoking, specific bacteria and inflammatory cytokines), suggests that more extensive studies with larger patient groups should be undertaken to analyse the relevance of the *MMP1-1607* SNP in the pathogenesis of periodontitis.

In fact, in addition to the influence of host genetic background, factors such as specific pathogens and cytokines may also account for the variance of MMP-1 levels in periodontal tissues (Chang et al. 2002, Garlet et al. 2004, Verstappen & Von den Hoff 2006, Andrian et al. 2007, Zhou & Windsor 2007, Mercer et al. 2009). Typical periodontopathogens, such as *P. gingivalis*, *T. denticola*, *T. forsythia* and *A. actinomycetemcomitans*, are described as inducers of MMP expression in vitro and in vivo (Tiranathanagul et al. 2004, Bodet et al. 2006, Feng & Weinberg 2006, Garlet et al. 2006, Zhou & Windsor 2006, Andrian et al. 2007, Zhou & Windsor 2007, Kou et al. 2008). Furthermore, the production of pro-inflammatory cytokines such as TNF-α and IL-1β in response to the periodontopathogens can also contribute towards increasing the levels of MMP-1 in periodontal lesions (Nishikawa et al. 2002, Graves & Cochran 2003, Garlet et al. 2004).

However, because the putative influences of the microbial, inflammatory and genetic factors have been investigated independently and in vitro, their exact individual and/or combined contribution to the determination of MMP-1 levels in vivo remain unknown. Therefore, given the important role of MMP-1 of a series of physiological and pathological processes, including the perio-

dontal diseases, the aim of the present study was to evaluate the contribution of genetic (*MMP1-1607* SNP), microbial and inflammatory (TNF-α and IL-1β) factors to the modulation of MMP-1 mRNA levels in vivo and in vitro, and its possible association with the periodontitis outcome.

Materials and Methods

Study population and clinical examination

Patients and controls (clinical features summarized in Table 1), from the south-eastern of Brazil, scheduled for treatment at the Dentistry School of University of Ribeirão Preto (UNAERP), signed a consent form that was approved by an Institutional Review Board, received supragingival prophylaxis and were subjected to anamnesis and to clinical [scored for bleeding on probing (BOP); probing depth (PD); clinical attachment loss (CAL)] and radiographic examination, and were then categorized into control (C) or chronic periodontitis (CP) groups (as described previously in the *Journal of Clinical Periodontology* and *Infection and Immunity*). Exclusion criteria comprised patients who did not give informed consent; patients with a significant medical history indicating evidence of known systemic modifiers of periodontal disease; or those who had been subjected to periodontal therapy in the previous 2 years, as described previously (Garlet et al. 2004, Ferreira et al. 2008). Smokers were specifically excluded. The C group ($N = 190$) comprised of subjects presenting gingival tissues that were clinically health (low scores of BOP – under 10% of the sites; no sites with PD > 3 mm or presenting CAL > 1 mm) scheduled for restorative dentistry procedures. After the diagnostic phase, C subjects received oral hygiene instructions and supragingival prophylaxis. CP patients ($N = 178$) had moderate to advanced PD (at least one tooth per sextant with PD > 6 mm and CAL > 3 mm, and radiographic evidence of extensive bone loss). After the diagnostic phase, CP patients, presenting moderate to advanced PD (i.e. at least one tooth per sextant with PD > 6 mm and CAL > 3 mm, and radiographic evidence of extensive bone loss), received basic periodontal therapy, which consisted in oral hygiene instruction, supragingival prophylaxis, scaling and root planing.

Sample collection

Epithelial buccal cells were sampled from inner cheek buccal mucosa scrapping after a mouthwash with 3% glucose of all C ($N = 190$) and CP ($N = 178$) subjects. Biopsies of gingival tissue of the C subjects ($N = 104$), one sample from each patient, were taken from sites that showed no BOP, PD < 3 mm and CAL < 1 mm during surgical procedures due to aesthetics, orthodontic and prosthetic reasons, and comprised junctional epithelium, gingival crevicular epithelium and connective gingival. The C subjects sampled presented the following characteristics: 50f/54m, age: 39.2 ± 9.94 years, 85/81.73% Caucasoids and 19/18.27% Afro-Americans/mulattos, mean PD: 2.50 ± 0.29, sampled site PD: 2.15 ± 0.76, sampled site CAL: 0, mean BOP: 5.00 ± 2.37%, sampled site BOP sites: 0%, all of them statistically similar to the whole CP group. Biopsies of gingival tissue of the CP patients ($N = 106$), one sample from each patient, were taken from the gingival margin to the bottom of the pocket, comprising junctional epithelium, periodontal pocket epithelium and connective gingival or granulation tissue, during surgical therapy of the sites that exhibited persistent BOP and higher PD 3–4 weeks after the basic periodontal therapy (non-responsive sites), as described previously (Ferreira et al. 2008). The CP patients sampled presented the following characteristics: 52f/54m, age: 46.5 ± 7.43 years, 84/79.24% Caucasoids and 22/20.75% Afro-Americans/mulattos, mean PD: 4.85 ± 0.86, sampled site PD: 7.05 ± 1.14, sampled site CAL: 4.23 ± 1.35, mean BOP: 62.13 ± 11.54%, sampled site BOP sites: 100%, all of them statistically similar to the whole CP group.

Table 1. Clinical features and frequencies of *MMP1-1607* single-nucleotide polymorphism (SNP) in control (C) and chronic periodontitis (CP) individual groups (mean ± SD)

	C ($N = 190$)	CP ($N = 178$)	p value
N and gender distribution	91f/99m	87f/91m	0.9169*
Age	41.5 ± 9.66	45.3 ± 8.63	0.0646†
Ethnic group	n (%)	N (%)	
Caucasoid	163 (85.78%)	149 (83.70%)	
Afro-American/Mulatto	27 (14.21%)	29 (16.29%)	0.6635*

Clinical parameters	value ± SD	value ± SD	
Probing depth (mean)	2.43 ± 0.33	4.79 ± 0.84	<0.001†
Probing depth (site)	2.08 ± 0.69	7.01 ± 1.17	<0.001†
Attachment loss (mean)	0.61 ± 0.16	3.09 ± 0.31	<0.001†
Attachment loss (site)	0.00 ± 0.00	4.23 ± 1.35	–
% BOP sites (mean)	5.15 ± 2.80	65.28 ± 13.94	<0.001†

MMP1-1607 genotypes	C ($N = 190$)	CP ($N = 178$)	p value*
1G/1G	65 (34.21)	46 (25.84)	
1G/2G	71 (31.36)	81 (45.50)	$p = 0.0760$, OR = 1.612, CI = 0.983–2.643
2G/2G	54 (28.42)	51 (28.65)	$p = 0.3596$, OR = 1.335, CI = 0.779–2.285
1G/2G+2G/2G	125 (65.78)	132 (74.15)	$p = 0.1022$, OR = 1.492, CI = 0.951–2.340

MMP1-1607 alleles	C ($n = 380$)	CP ($n = 356$)	
1G	201 (52.89)	173 (48.59)	
2G	179 (47.10)	183 (51.40)	$p = 0.2748$, OR = 1.188, CI = 0.889–1.587

*χ^2-test.
†Unpaired t-test.
OR, odds ratio; CI, confidence interval; BOP, bleeding on probing.

Analysis of *MMP1-1607* SNP

DNA was extracted from epithelial buccal cells (sampled from inner cheek buccal mucosa scrapping after a mouthwash with 3% glucose) with a sequential phenol/chloroform solution and precipitated with salt/ethanol solu-

tion, as described previously (Trevilatto & Line 2000). *MMP1-1607* (rs: 1799750) genotyping was performed as described previously (de Souza et al. 2003). A 35-cycle standard polymerase chain reaction (PCR) was performed with 200 ng of sample DNA and specific primers (Table 2) (de Souza et al. 2003). A 15 μl aliquot of PCR product was subjected to the restriction fragment length polymorphism with 3 U of XmnI (20 U/ml) (New England Biolabs Inc., Beverly, MA, USA) at 37°C overnight, and the products were resolved and separated on 2% agarose gel stained with SYBRsafe (Invitrogen Life Technologies, Carlsbad, CA, USA). The site that allows endonuclease activity is present in MMP-1 1G (but not 2G) at the polymorphism site, resulting in two fragments of 89 and 29 bp.

Real-time PCR reactions – mRNA quantification

The extraction of total RNA from periodontal tissues samples, performed with Trizol reagent (Invitrogen), and the cDNA synthesis were accomplished as described previously (Ferreira et al. 2008). Real-time PCR mRNA or DNA analyses were performed in an MiniOpticon system (BioRad, Hercules, CA, USA), using SybrGreen MasterMix (Invitrogen), specific primers and 2.5 ng of cDNA in each reaction, as described previously (Ferreira et al. 2008, Trombone et al. 2008). The primer sequences and reaction properties are depicted in Table 2. The standard PCR conditions were 95°C (10 min.), and then 40 cycles of 94°C (1 min.), 56°C (1 min.) and 72°C (2 min.), followed by the standard denaturation curve. Negative controls without cDNA and without the primer/probe sets were also performed. Calculations for determining the relative levels of gene expression were made from triplicate measurements of the target gene, with normalization to β-actin in the sample, using the cycle threshold (C_t) method and the $2^{\Delta\Delta C_t}$ equation, as described previously (Ferreira et al. 2008, Trombone et al. 2008).

Real-time PCR reactions – bacterial DNA quantification

In order to allow the detection of *P. gingivalis*, *T. forsythia*, *T. denticola* and *A. actinomycetemcomitans*, periodontal crevice/pocket biofilm samples were collected with sterile paper point ISO #40 from the same site biopsied before the surgical procedure (Nonnenmacher et al. 2004). Bacterial DNAs were extracted from plaque samples by the DNA Purification System (Promega, Madinson, WI, USA) (Ferreira et al. 2008). Real-time PCR mRNA or DNA analyses were performed in an MiniOpticon system (BioRad), using SybrGreen MasterMix (Invitrogen), specific primers and 5 ng of DNA in each reaction (Trombone et al. 2008). The primer sequences and reaction properties are depicted in Table 2. The standard PCR conditions were 95°C (10 min.), and then 40 cycles of 94°C (1 min.), 56°C (1 min.) and 72°C (2 min.), followed by the standard denaturation curve. The positivity to bacteria detection and the bacterial counts in each sample were determined based on the comparison with a standard curve comprised by specific bacteria DNA (10^9–10^{-2} bacteria) and negative controls, similar to that described previously (Trombone et al. 2008), and then adjusted for sample dilution in the assay to determine the bacterial copy numbers in each site (i.e. absolute load), and subsequently normalized by the PD of the site (i.e. normalized load). The sensibility range of bacteria detection and quantification of our real-time PCR technique was of 10^1–10^8 bacteria to each of the four periodontopathogens tested.

Table 2. Primer sequences and reaction properties

Target	Sense and anti-sense sequences	t_A (°C)	t_M (°C)	bp
MMP1-1607 (rs: 1799750)	TCGTGAGAATGTCTTCCCATT TCTTGGATTGATTTGAGATAAGTGAAATC	55	–	29/89
MMP-1	TGGACCTGGAGGAAATCTTGC AGAGTCCAAGAGAATGGCCGA	58	79	155
TNF-α	AAGCCTGTAGCCCATGTTGT CAGATAGATGGGCTCATACC	56	79	330
IL-1β	GGAAGATTCTGAAGAAGAGAC TGAGATTTTTAGAGTAACAGG	58	79	329
β-actin	ATGTTTGAGACCTTCAACA CACGTCAGACTTCATGATGG	56	75	195
Porphyromonas gingivalis	TACCCATCGTCGCCTTGGT CGGACTAAAACCGCATACACTTG	60	84	126
Treponema denticola	AGAGCAAGCTCTCCCTTACCGT TAAGGGCGGCTTGAAATAATGA	59	80	105
Tannerella forsythia	GGGTGAGTAACGCGTATGTAACCT ACCCATCCGCAACCAATAAA	59	79	127
Aggregatibacter actinomycetemcomitans	ATGCCAACTTGACGTTAAAT AAACCCATCTCTGAGTTCTTCTTC	60	78	157

t_A, annealing temperature; t_M, melting temperature; bp, base pairs of amplicon size; MMP-1, matrix metalloproteinase-1; TNF-α, tumour necrosis factor-α; IL-1β, interleukin-1β.

Cell culture

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood from 24 systemically and periodontally healthy subjects (12 subjects with the 1G/1G genotype and 14 presenting 2G allele: 5 1G/2G and 9 2G/2G) by density gradient centrifugation with Ficoll (Invitrogen), as described previously (Ho et al. 2008). CD14+ monocytes were purified from fresh PBMCs with anti-CD14 magnetic beads (MiltenyiBiotec, Bergisch, Gladbach, Germany), as recommended by the manufacturer and as described previously (Ho et al. 2008). The purity of monocytes was between 95% and 97% as verified by FACS. Monocytes were cultured at 5×10^5 cells/ml in RPMI medium (Invitrogen) supplemented with 10% FBS (HyClone, Waltham, MA, USA), and subjected to single or repeated (2 or 3, with 24-h intervals) *P. gingivalis* or *Escherichia coli* lipopolysaccharide (LPS; 1, 10 or 100 ng/ml) (Sigma-Aldrich, St. Louis, MO, USA) or cytokine (TNF-α+IL-1β: 0.5, 5 or 50 ng/ml of each cytokine) (BD Biosciences Pharmingen, San Jose, CA, USA) stimulation. The doses and the repeated stimulation protocol were defined based on previous studies (Ardans et al. 2002, Kiszel et al. 2007, Sundararaj et al. 2008) and preliminary experiments, to achieve a low, intermediate and intense degree of stimuli to the cells. After 12-h poststimulation, the cells were collected and subjected to RNA extraction as described above.

Statistical analysis

The significance of the differences in the observed frequencies of the study polymorphism was assessed by the χ^2-test, and the risk associated with genotypes/alleles was calculated as the odds ratio (OR) with 95% confidence intervals (CIs). Analysis of MMP-1 expression and bacterial load when performed only between two groups and in vitro data analysis between 1G and 2G genotype groups within the same concentration and stimulation protocol were performed with a *t*-test. Analysis of possible differences between the genotype subgroups of C and CP groups was performed by ANOVA, followed by Tukey's test, and different letters in the figure indicate statistical significance among the groups investigated. Multiple logistic and linear regression analyses were performed to evaluate the possible association between the variables. Values of $p < 0.05$ were considered statistically significant.

Results

MMP1-1607 SNP frequency analysis in the C and CP groups

The subject sample included in this study had a similar composition of male and female subjects (Table 1). In terms of ethnic status, Caucasians were prevalent compared with Afro-Americans/mulatto individuals; however, no further analysis was performed based on this classification in view of the high genetic miscegenation of the Brazilian population (Parra et al. 2003). The frequencies of *MMP1-1607* SNP genotypes and alleles were similar in the CP and C groups (Table 1), and were also found to be similar to that reported for the Brazilian population in a previous study (Astolfi et al. 2006). The distribution of genotypes in both groups was found to be in Hardy–Weinberg equilibrium.

Association of *MMP1-1607* with MMP-1 mRNA expression and with clinical parameters

In order to evaluate the putative functionality of *MMP1-1607* SNP, we next correlated it with MMP-1 mRNA expression in healthy and diseased gingival tissues (Fig. 1). Our data showed a weak MMP-1 expression in periodontal tissues of the C subjects, while a significantly stronger expression was evidenced in the tissues harvested from CP patients. When analysing the possible association between MMP-1 mRNA levels and the *MMP1-1607* SNP genotypes in the C group, we found that polymorphic allele 2G (1G/2G and 2G/2G genotypes) carriers presented a significantly higher MMP-1 mRNA expression when compared with the 1G/1G genotype group. Conversely, in the CP group, no differences were found in MMP-1 expression between the different genotype groups. As expected, the scores of the clinical parameters of disease severity were significantly higher in the CP than in the C group (Table 1), but no associations were found between the clinical parameters and the *MMP1-1607* genotypes in both C and CP groups (data not shown).

Association of periodontopathogens and cytokines with MMP-1 mRNA expression in vivo

Given the importance of microbial factors in the aetiology of periodontal disease, we next correlated its presence and load with the intensity of MMP-1 mRNA expression. The frequency (Table 3) and the absolute and normalized load (Fig. 2) of the periodontopathogens *P. gingivalis*, *T. denticola and T. forsythia* (which comprise the red complex), and *A. actinomycetemcomitans* were significantly more prevalent in the CP than in the C group. Similarly, the levels of bacterial 16S DNA were strikingly higher in the CP when compared with the C (data not shown). No associations were found between the presence of periodontopathogens and MMP-1 expression in the C group (Fig. 3). Conversely, red complex periodontopathogen detection was associated with a significantly higher expression of MMP-1 mRNA, and *P. gingivalis* ($r^2 = 0.0589$, $p = 0.0008$), *T. forsythia* ($r^2 = 0.0449$, $p = 0.0033$) and *T. denticola* ($r^2 = 0.1161$, $p < 0.0001$) absolute loads were also positively correlated with MMP-1 mRNA expression in diseased tissues. The normalized bacterial load was also found to be similarly correlated with the levels of MMP-1 in periodontal lesions (data not shown). The samples positive to *A. actinomycetemcomitans* detection presented a higher MMP-1 expression than the negative ones, but this difference was not statistically significant. No correlations were found between *A. actinomycetemcomitans* load (both absolute and normalized loads) and MMP-1 levels ($r^2 = 0.0101$, $p = 0.1697$). Because previous studies correlated periodontopathogens with inflammatory cytokine production (Claudino et al. 2008, Ferreira et al. 2008), and inflammatory cytokines can up-regulate MMP-1 expression, we next investigated possible correlations between these factors' expressions. Our results demonstrated that IL-1β ($r^2 = 0.1088$, $p = 0.0107$) and TNF-α ($r^2 = 0.0657$, $p = 0.0080$) were positively correlated with MMP-1 mRNA expression, while no association was found between MMP-1 and IL-6 (data not shown).

MMP1-1607 genotype and periodontopathogens *versus* MMP-1 mRNA expression

Because the periodontopathogens and *MMP1-1607* SNP could play overlap-

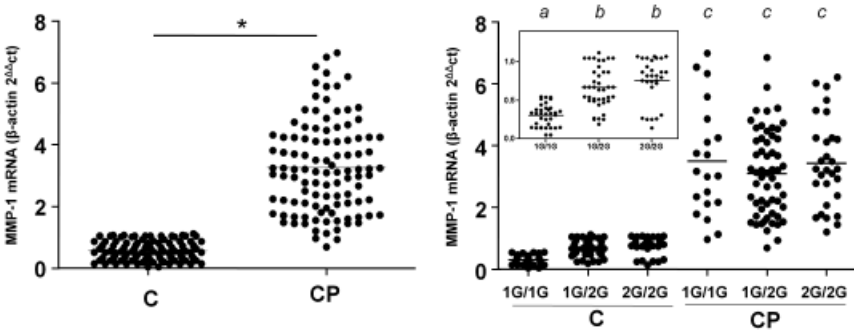

Fig. 1. MMP1-1607 single-nucleotide polymorphism (SNP) and its association with matrix metalloproteinase-1 (MMP-1) mRNA levels in control (C) subjects and chronic periodontitis (CP) patients. Total RNA was extracted from gingival tissues of C ($N = 104$) and CP ($N = 106$) patients, and levels of MMP-1 mRNA were determined by real-time polymerase chain reaction (PCR; with normalization to β-actin using the C_t method) and the results are presented as expression of the individual mRNAs (with normalization to β-actin using the C_t method). The box depicts the C group with a different *Y*-axis scale. $^*p < 0.05$: *t*-test; one-way ANOVA, followed by Tukey's test: different letters indicate statistical significance ($p < 0.05$) among the experimental groups.

Table 3. Frequencies of periodontopathogens detection in control subjects (C) and chronic periodontitis (CP) patients regarding their *MMP1-1607* genotype

Bacteria	C ($N = 104$)				CP ($N = 106$)				χ^2-test
	negative (*N*/%)		positive (*N*/%)		negative (*N*/%)		positive (*N*/%)		
Porphyromonas gingivalis	91	87.50	13	12.50	39	36.79	67	63.20	$p < 0.0001$, OR = 12.026, CI: 5.956–24.282
1G/1G	28	26.92	7	6.73	6	5.66	14	13.20	
1G/2G+2G/2G	63	60.57	6	5.76	33	31.13	53	50.00	
	$p = 0.1824$, OR = 0.3810, CI: 0.1173–1.237				$p = 0.6585$, OR = 0.6883, CI: 0.2407–1.968				
Treponema denticola	94	90.38	10	9.61	48	45.28	58	54.71	$p < 0.0001$, OR = 11.358, CI: 5.333–24.131
1G/1G	30	28.84	5	4.80	7	6.60	13	12.26	
1G/2G+2G/2G	64	61.53	5	4.80	41	38.67	45	42.45	
	$p = 0.4245$, OR = 0.4088, CI: 0.1260–1.744				$p = 0.4376$, OR = 0.5910, CI: 0.2149–1.626				
Tannerella forsythia	95	91.34	9	8.65	45	42.45	61	57.54	$p < 0.0001$, OR = 14.309, CI: 6.528–31.364
1G/1G	31	29.80	4	3.84	6	5.66	14	13.20	
1G/2G+2G/2G	64	61.53	5	4.80	39	36.79	47	44.33	
	$p = 0.7280$, OR = 0.6055, CI: 0.1518–2.415				$p = 0.3174$, OR = 0.5165, CI: 0.1813–1.471				
Red complex	77	74.03	27	25.96	22	20.75	84	79.24	$p < 0.0001$, OR = 10.889, CI: 5.728–20.701
1G/1G	22	21.15	13	12.50	6	5.64	18	16.98	
1G/2G+2G/2G	55	52.88	14	13.46	16	15.10	66	62.26	
	$p = 0.1062$, OR = 0.4308, CI: 0.1747–1.062				$p = 0.5741$, OR = 0.3667, CI: 0.07825–1.718				
Aggregatibacter actinomycetemcomitans	97	93.26	7	6.73	89	83.96	17	16.03	$p = 0.0571$, OR = 2.647, CI: 1.048–6.683
1G/1G	34	32.69	1	0.96	13	12.26	7	6.60	
1G/2G+2G/2G	63	60.57	6	5.76	76	71.69	10	9.43	
	$p = 0.4785$, OR = 3.238, CI: 0.3741–28.028				$p = 0.0253$, OR = 0.2449, CI: 0.07884–0.7574				

χ^2-test: performed to access the odds of periodontopathogens detection in the C and CP groups and also in the different genotype subgroups (1G/1G *versus* 1G/2G+2G/2G); subjects were considered red complex positive when at least one of the red complex species was detected.
OR, odds ratio; CI, confidence interval.

ping roles in the determination of MMP-1 mRNA levels, we next analysed MMP-1 expression in subgroups clustered regarding their genotype and the presence/absence of target bacteria. This analysis confirmed the previous results, demonstrating that in diseased tissues, both red complex periodontopathogens and *A. actinomycetemcomitans* were associated with increased MMP-1 mRNA levels, irrespective of the *MMP1-1607* genotype (Fig. 4). Similar results were found when *P. gingivalis*, *T. denticola* and *T. forsythia* were analysed individually (data not shown). In contrast, no differences were found in the frequency (Table 2) or in the load (data not shown) of the periodontopathogens investigated in the different *MMP1-1607* genotypes within the CP group. Multiple logistic regression analysis demonstrated that MMP-1 expression is age ($p = 0.516$, OR = 0.8, CI = 0.8–1.1), race ($p = 0.477$, OR = 1.0, CI = 0.5–1.9) and gender ($p = 0.802$, OR = 1.1, CI = 0.5–2.6) independent.

MMP1-1607 versus LPS and cytokine stimulation in vitro

The LPS and cytokine (TNF-α+IL-1β) stimulation of both 1G (1G/1G genotype) and 2G (1G/2G and 2G/2G) macrophages resulted in a dose-response MMP-1 mRNA expression (Fig. 5). Regarding the single-stimulation protocol, low and intermediate stimuli with both *P. gingivalis* LPS and cytokine resulted in a genotype-dependent response, where 2G cells presented a higher expression of MMP-1, while an intense/strong stimulus resulted in a high MMP-1 expression irrespective of the cell genotype. Regarding the repeated stimulation protocol, the cell genotype only resulted in higher MMP-1 expression under low LPS stimuli, while the other LPS concentrations and cytokine stimulation resulted in similar MMP-1 expression by 1G and 2G macrophages. *E. coli* LPS stimulation resulted in similar patterns of macrophage MMP-1 mRNA expression regarding LPS concentrations and single/repeated stimulation (data not shown), but the overall MMP-1 mRNA expression in response to *P. gingivalis* LPS was found to be significantly higher. A 7 × cell stimulation protocol resulted in a response similar to that seen to 3 × stimulation (data not shown). For all the protocols tested, no significant differences were found between 1G/2G and 2G/2G genotypes (data not shown).

Discussion

Matrix metalloproteinases play a role in both the physiological and the pathological degradation of extracellular and bone matrix proteins in the periodontal environment (Reynolds et al. 1994, Hannas et al. 2007). While MMP-1 levels are relatively low in healthy periodontal tissues, an increase in its levels is associated with tissue destruction (Dean et al. 1989, Murphy et al. 1991, Reynolds 1996, Nawrocki et al. 1997). In accordance with previous

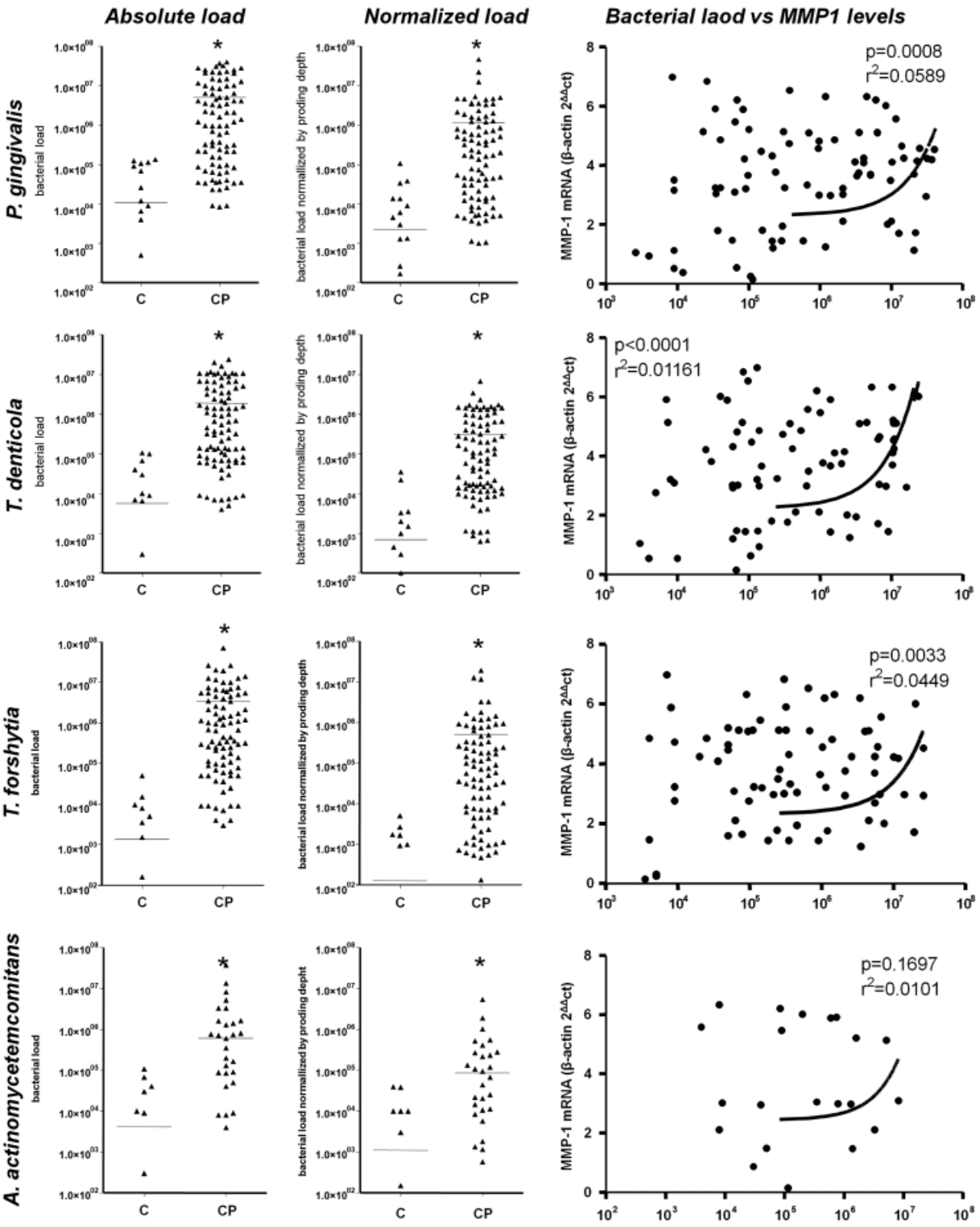

Fig. 2. Bacterial load and its association with the expression of matrix metalloproteinase-1 (MMP-1) mRNA in controls (C) and chronic periodontitis (CP) patients. Total RNA was extracted from gingival tissues of C ($N = 104$) and CP ($N = 106$) patients, and levels of MMP-1 mRNA were determined by real-time polymerase chain reaction (PCR; with normalization to β-actin using the C_t method). DNA was extracted from biofilm samples collected from the same site biopsied, and the load of the periodontopathogens *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Actinobacillus actinomycetemcomitans* was determined by real-time PCR. The graphs depict the absolute bacterial load in sites sampled from the C and CP groups ($^*p < 0.05$, *t*-test) the relative bacterial load (bacterial load normalized by crevice/pocket depth) in the C and CP groups ($^*p < 0.05$, *t*-test), and the association between the periodontopathogen load and MMP-1 mRNA levels in the CP samples (values of r^2 and p of linear regression analysis are indicated in the graphs).

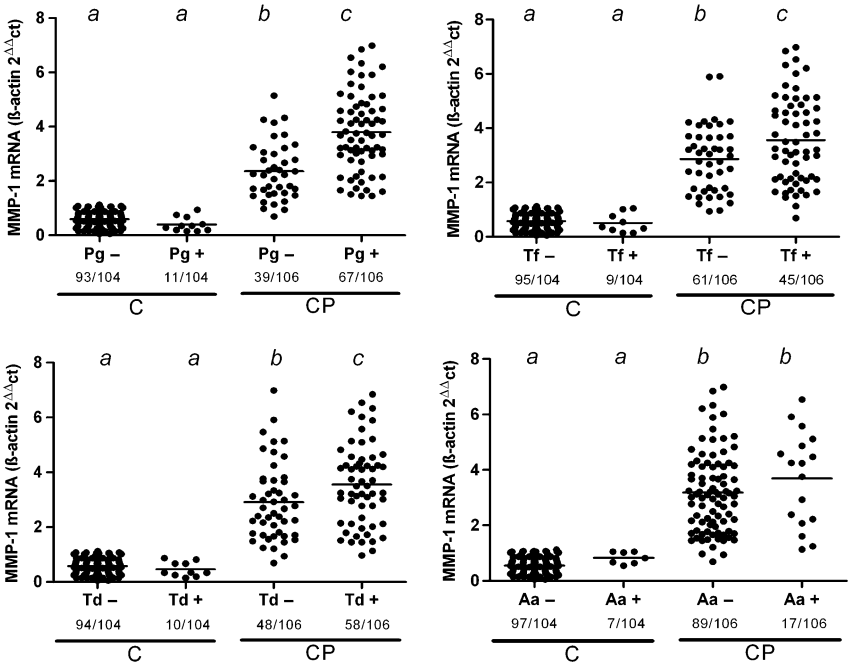

Fig. 3. Association between the presence of periodontopathogens and the expression of matrix metalloproteinase-1 (MMP-1) mRNA in controls (C) and chronic periodontitis (CP) patients. Total RNA was extracted from gingival tissues of C ($N = 104$) and CP ($N = 106$) patients, and levels of MMP-1 mRNA were determined by real-time polymerase chain reaction (PCR; with normalization to β-actin using the C_t method). DNA was extracted from biofilm samples collected from the same site biopsied, and the identification of the presence of the periodontopathogens *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Actinobacillus actinomycetemcomitans* periodontal pockets was performed by real-time PCR. The graphs depict the expression of MMP-1 in C and CP patients regarding their positivity to each bacteria detection; one-way ANOVA, followed by Tukey's test: different letters indicate statistical significance ($p < 0.05$) among the experimental groups.

studies (Reynolds et al. 1994, Hannas et al. 2007), our data demonstrate a significantly higher MMP-1 expression in the tissues of CP patients when compared with the C patients, and a considerable degree of variance in individual MMP-1 mRNA levels. Therefore, because individual variations in the MMP-1 levels are thought to have a significant impact on periodontitis outcome, we next investigated the influence of host genetic background, inflammatory cytokines and specific periodontopathogens on the determination of MMP-1 mRNA levels.

From the genetic association point of view, no differences were found in the frequency of *MMP1-1607* SNP between the C and the CP groups, which suggests the lack of association with disease susceptibility. However, we must consider that PD, a complex trait multifactorial disease, presents a series of characteristics that impair the classic genetic association analysis. The major fraction of the subjects (around 70%) is susceptible to the development of the disease, but the existence of periodontally healthy subjects does not necessarily imply a genetic resistance to the disease development, but may only reflect the control of the aetiological factors of disease (Shapira et al. 2005, Huynh-Ba et al. 2007, Claudino et al. 2008, Ferreira et al. 2008), or in other words, the C group comprising periodontally healthy subjects invariably includes a high number of subjects genetically prone to develop periodontitis but with proper oral hygiene. Additionally, distinct ethnics of the subjects investigated, differences in the criteria to select C and CP groups and, mainly, the observance (or not) of covariates with potential overlapping effects may hinder their interpretation (Shapira et al. 2005, Huynh-Ba et al. 2007, Claudino et al. 2008, Ferreira et al. 2008). Therefore, it is difficult to clearly state or rule out that a given SNP is associated with periodontitis development based only on SNP frequency data, and indeed, the genetic association studies regarding PD (to a number of genes, including MMP1) are usually highly controversial (Holla et al. 2004, Itagaki et al. 2004, Cao et al. 2005, 2006, Astolfi et al. 2006, Pirhan et al. 2008, Ustun et al. 2008).

Alternatively, from a functional or a mechanistic point of view, the modulation of a biological phenomenon by a given SNP can point to a positive association with a given disease irrespective of the genotype frequency, as demonstrated in previous studies (Shapira et al. 2005, Huynh-Ba et al. 2007, Claudino et al. 2008, Ferreira et al. 2008). Specifically for *MMP1-1607*, an SNP that creates a binding site for transcription factors, the expected primary functional effect is the modulation of the transcriptional activity of MMP-1 (Kanamori et al. 1999, Ye 2000, Pirhan et al. 2008). Indeed, our results demonstrate that the presence of the *MMP1-1607* 2G allele was associated with increased MMP-1 mRNA expression in healthy periodontal tissues (i.e. control subjects). Accordingly, studies demonstrate that the *MMP1-1607* polymorphism affects gene transcription. The *MMP1-1607* 2G allele, together with an adjacent adenosine, creates a core binding site (5′-GGA-3′) for transcription factors immediately adjacent to an AP-1 site, causing a significant increase in the transcription activity in vitro (Kanamori et al. 1999, Ye 2000, Sternlicht & Werb 2001).

However, on analysing the possible association of *MMP1-1607* with MMP-1 variance in diseased periodontium, no statistical differences were found in MMP-1 mRNA levels within the *MMP1-1607* genotypes, suggesting that this SNP may not play a relevant role in the development of periodontal disease. According to this hypothesis, the *MMP1-1607* SNP genotype was not associated with the clinical parameters of disease severity in our sample. However, observance (or not) of covariates (specific bacteria, inflammatory cytokines, smoking) with potential overlapping effects is mandatory to unravel a possible small, but significant, functional role of the SNP investigated (Shapira et al. 2005, Huynh-Ba et al. 2007, Claudino et al. 2008, Ferreira et al. 2008). Indeed, previous studies hypothesize that these same covariates may contribute towards regulation of MMP-1 expression in diseased periodontium rather than gene polymorphisms (Chang et al. 2002).

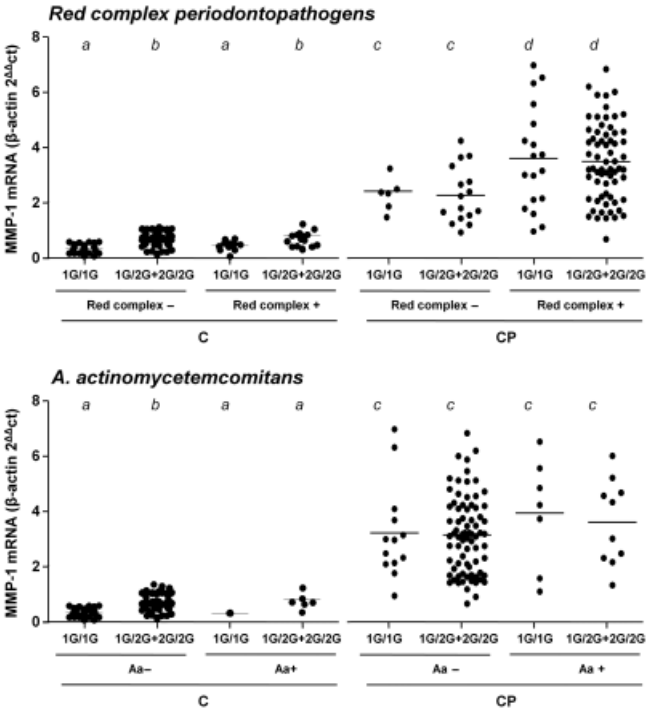

Fig. 4. Quantitative assessment of matrix metalloproteinase-1 (MMP-1) mRNA expression in the presence or absence of *Actinobacillus actinomycetemcomitans* and red complex periodonthogens associated with the genotypes. *MMP1-1607* single-nucleotide polymorphism (SNP) genotype of controls (C, $N = 104$) and chronic periodontitis (CP, $N = 106$) patients was determined by RFLP, and levels of MMP-1 mRNA, and the detection of the periodontopathogens in periodontal pocket was performed by real-time polymerase chain reaction (PCR). The graphs depict the expression of MMP-1 in the presence or absence of *Actinobacillus actinomycetemcomitans* and red complex periodontopathogens in the C and CP patients regarding their *MMP1-1607* SNP genotype. One-way ANOVA, followed by Tukey's test: different letters indicate statistical significance ($p < 0.05$) among the experimental groups.

Therefore, we next investigated the possible contribution of known periodontopathogens to MMP-1 modulation. Our results showed that the frequencies and load of periodontopathogens were strikingly higher in the CP group, where the presence of *P. gingivalis*, *T. forsythia* or *T. denticola* was associated with increased MMP-1 mRNA expression. Accordingly, *P. gingivalis* increases the mRNA expression of MMP-1 (Zhou & Windsor 2006, 2007, Andrian et al. 2007, Kou et al. 2008), while *T. denticola* and *T. forsythia* have been associated with increased MMP activity in vitro (Ding et al. 1997, Bodet et al. 2006, Zhou & Windsor 2007). In addition, the red complex bacteria were associated with higher scores of disease severity parameters (data not shown), in accordance with previous studies (Feng & Weinberg 2006, Claudino et al. 2008, Ferreira et al. 2008). Our results also demonstrate that *A. actinomycetemcomitans*, described to induce MMP production in vitro and in vivo (Tiranathanagul et al. 2004, Bodet et al. 2006, Garlet et al. 2006), was only associated with a trend towards a higher MMP-1 expression, which probably did not reach statistical significance due to the low frequency of this bacteria in our sample. At this point, it is important to consider that the evaluation of the presence and load of periodontopathogens by means of real-time PCR presents a high sensitivity and specificity compared with the culture technique, which justifies its use as a diagnostic and investigational tool, even in spite of not providing information such as microbial viability and antibiotic susceptibility (Sanz et al. 2004, Jervoe-Storm et al. 2005, Atieh 2008).

Interestingly, when both genetic and microbial variants were analysed simultaneously to avoid possible overlapping effects, we found an interesting scenario: in the control group, the *MMP1-1607* 2G allele was associated with increased MMP-1 expression irrespective of the presence of red complex periodontopathogens, while in diseased tissues, periodontopathogens were associated with increased MMP-1 expression independent of the *MMP1-1607* genotype. It is also important to consider that the frequency and load of periodontopathogens were similar in all *MMP1-1607* genotypes within the CP group, excluding therefore a possible additional puzzling factor. Interestingly, even the patients negative for the classic periodontopathogens presented a high MMP-1 expression, which is also independent of the *MMP1-1607* genotype. This increased expression (when compared with controls) is probably due to the presence of other Gram-negative bacterial species, less virulent than the classic periodontopathogens but also able to contribute to periodontitis development to a lesser extent (Feng & Weinberg 2006). Based on our in vivo data and considering the genetic and microbial influence over MMP-1 expression, we can identify distinct subgroups: subjects exposed to a low microbial challenge where the effect of the *MMP1-1607* genotype is relevant to the determination of MMP-1 levels (i.e. control subjects), and subjects in whom the intermediate and intense microbial challenge, considering the overall bacterial load and the presence of specific periodontopathogens, plays a major role over MMP-1 expression irrespective of the *MMP1-1607* genotype.

Therefore, we hypothesize that the prolonged stimulation of host cells by the periodontopathogens, and by the inflammatory mediators characteristically induced by them, may lead to a high MMP-1 expression independent of the *MMP1-1607* genotype. In order to investigate this possibility, we designed in vitro experiments using low, intermediate and strong LPS and inflammatory cytokine stimuli that could mimic the clinical situations found in the C and CP subjects. LPS is the main structural component of a Gram-negative bacteria cell wall, is the immunodominant antigen expressed by periodontopathogens (Wilson & Hamilton 1992, Feng & Weinberg 2006) and was described to induce MMP expressions through TLR signalling (Lai et al. 2003, Kasamatsu et al. 2005). In addition, TLR signalling also increases MMP-1 expression mediated by pro-inflammatory cytokines, such as TNF-α and IL-1β (Yamaji et al. 1995, Ardans et al. 2002, Nishikawa et al. 2002, Itoh et al. 2003, Rossa et al. 2007), cytokines whose levels

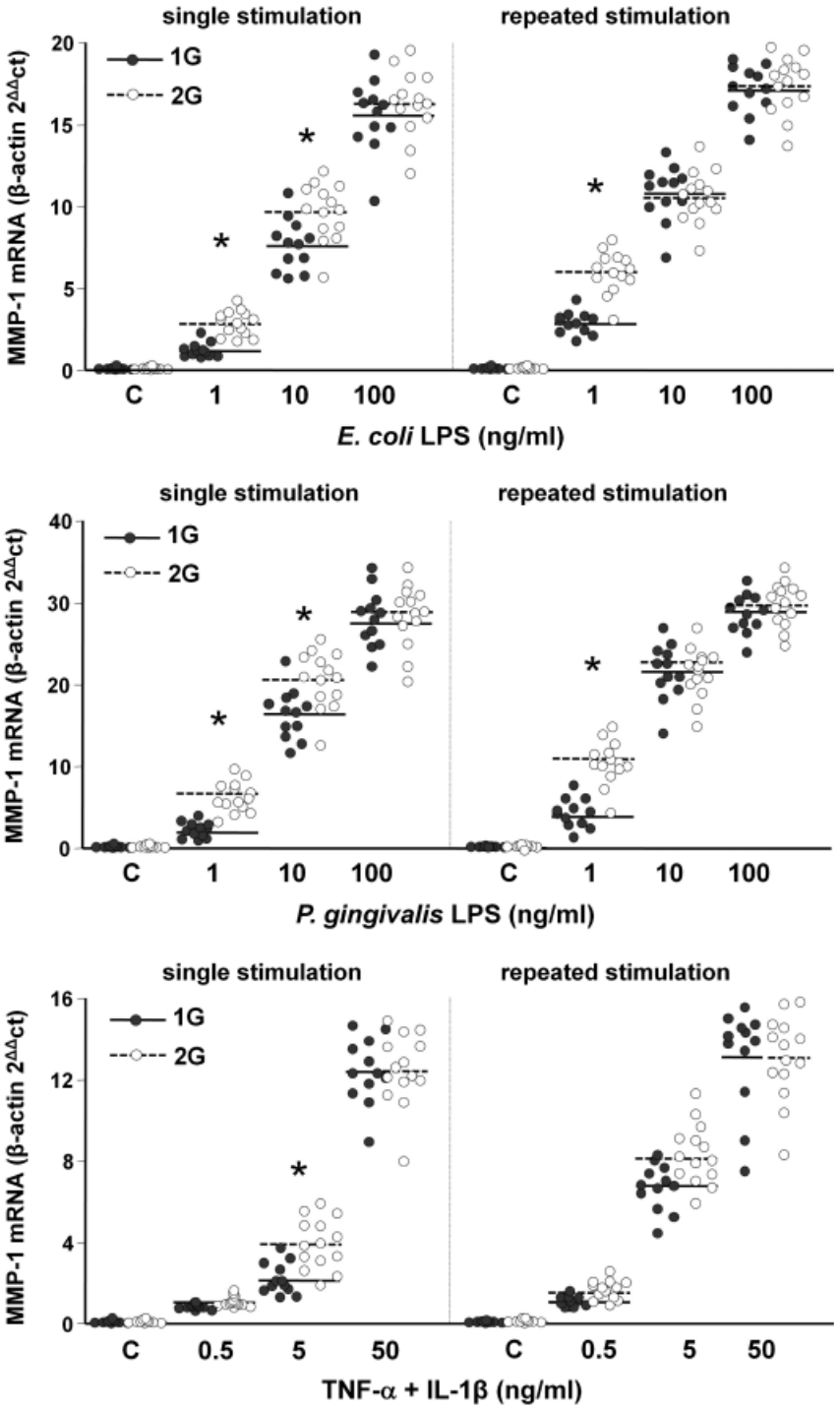

Fig. 5. Strong and repeated microbial and inflammatory stimuli overcome the effects of the *MMP1-1607* single-nucleotide polymorphism (SNP) genotype on matrix metalloproteinase-1 (MMP-1) expression in macrophages. Macrophages (5×10^5 cells/ml, obtained from 26 systemically and periodontally healthy donors fresh PBMCs purification with anti-CD14 magnetic beads) presenting *MMP1-1607* 1G (1G/1G, $N = 12$) or 2G polymorphic (1G/2G, $N = 5$; 2G/2G, $N = 9$) genotypes were cultivated in RPMI medium supplemented with 10% FBS, and subjected to single or repeated (3 ×, with 24-h intervals) *Porphyromonas gingivalis* LPS (1, 10 or 100 ng/ml) or cytokine (TNF-α+IL-1β; 0.5, 5 or 50 ng/ml of each cytokine) stimulation. After 12-h poststimulation, the cells were collected and the levels of MMP-1 mRNA were quantitatively measured by real-time polymerase chain reaction (PCR), and the results are presented as expression of the individual mRNAs (with normalization to β-actin using the C_t method). $^*p < 0.05$, *t*-test; 1G *versus* 2G within the same concentration and stimulation protocol.

were positively correlated with MMP-1 expression in our sample. Interestingly, the short-term and lower degree stimulation of macrophages, designed to parallel with the C group, exposed to a very low bacterial and inflammatory challenge, demonstrates the host genotype influence on MMP-1 mRNA levels, which is compatible with our data from healthy tissues. Alternatively, repeated and higher degree stimuli result in a higher MMP-1 expression irrespective of the genotype, similar to that seen in CP patients, where a massive and sustained accumulation of Gram-negative bacteria and inflammatory cytokines takes place. Interestingly, *P. gingivalis* LPS resulted in a pattern of macrophage response similar to that of *E. coli* LPS regarding the effect of the distinct concentration and stimulation protocol, except for the more robust MMP-1 expression induced by *P. gingivalis* LPS. This result reinforces the hypothesis that specific bacteria such as *P. gingivalis*, and its specific LPS, may result in a significant increase in host responsiveness (Zhou & Windsor 2006, 2007, Andrian et al. 2007, Kou et al. 2008, Barksby et al. 2009). At this point, it is very important to consider that the in vitro experiments were conducted with cells from systemically and periodontally healthy subjects, in order to avoid a possible priming influence of circulating cytokines in the subsequent stimulation. Still in this context, it is possible to consider that the repeated stimulation protocol may mimic this initial priming that takes place in periodontitis patients (Pussinen et al. 2004, 2007, Golub et al. 2006), which consequently would also contribute to a higher MMP-1 expression irrespective of the host *MMP1-1607* genotype. Therefore, we can conclude that the effect of the *MMP1-1607* genotype is dependent on the strength and extent of the stimuli: while under low-challenge stimulation, the SNP possibly facilitate/extend the action of transcription factors leading to higher MMP-1 transcription, a stronger signalling due to intense and sustained stimulation overcome the genetic predisposition, and high levels of MMP-1 are transcribed irrespective of the SNP.

Interestingly, studies demonstrate that highly dissimilar phenotypes can result from stimuli/SNPs interaction, including dose-dependent and -independent responses, additive, synergic or antagonic reactions (Pirmohamed & Park 2001, Dall'Ozzo et al. 2004, Shah 2005, Tai et al. 2005, Godfrey et al.

2007, Hu et al. 2007, Hashimoto et al. 2008). Specifically in PD, IL1B+3954 and red complex periodontopathogens present an additive effect over IL-1β expression (Claudino et al. 2008, Ferreira et al. 2008), while no additive effect was found between TNFA-308 and periodontopathogens, both factors being independently associated with TNF-α levels (Feng & Weinberg 2006, Claudino et al. 2008, Ferreira et al. 2008). Since IL-1β and TNF-α can modulate MMP-1 expression, as well as the expression of other MMPs relevant to periodontitis development, IL1B+3954 and TNFA-308 SNPs are potentially more relevant to PD outcome than *MMP1-1607*. Indeed, IL1B+3954 and TNFA-308 were associated with higher values of the clinical parameters of disease severity (Feng & Weinberg 2006, Claudino et al. 2008, Ferreira et al. 2008), while no associations were found with *MMP1-1607*. Another SNP potentially involved in the MMPs/tissue inhibitor of metalloproteinases (TIMPs) system regulation is the IL10-592, described to influence the levels of TIMP expression in periodontal tissues (Garlet et al. 2004, 2006, Claudino et al. 2008). Indeed, MMPs can also be regulated at multiple levels rather than transcriptionally (i.e. secretion, activation, inhibition), and TIMPs play a very important role in this system (Sternlicht & Werb 2001, Garlet et al. 2004, 2006, Claudino et al. 2008). Therefore, further studies are required to confirm whether increased levels of MMP-1 mRNA are indeed associated with increased MMP-1 activity in periodontal lesions, and also the role of different TIMPs in the determination of MMP-1 activity in vivo and in vitro. It is also important to consider that periodontitis is a complex trait disease, in which several other MMPs, periodontopathogens and immunoregulatory cytokines not considered in this investigation may also be involved in the modulation of disease outcome. An additional point to be considered is the timing of sample collection, because in this study only sites non-responsive to basic periodontal therapy were sampled for microbiological and molecular analysis. In view of the impossibility to determine the presence of active bone loss in a given periodontal pocket, non-responsive sites were intentionally sampled in order to maximize the number of potentially active periodontal lesions to be investigated. Indeed, based on the comparison of RANKL/OPG expression among non-responsive sites and sites with active or inactive alveolar bone resorption (induced by orthodontic forces), a previous study demonstrated that the majority of the non-responsive sites were compatible with the active bone resorption molecular profile (Menezes et al. 2008). Interestingly, unpublished data, generated using the same strategy described above to analyse the RANKL/OPG expression pattern, demonstrate that samples collected before the basic periodontal therapy comprise $<40\%$ of putative active bone resorption sites. Therefore, we must consider that the analysis of samples collected before the basic periodontal therapy may result in distinct levels of periodontopathogens and MMP-1 mRNA than in non-responsive sites.

Taken together, our results demonstrate distinct roles for genetic and microbial factors in the regulation of MMP-1 expression in healthy and diseased periodontal tissues. While the *MMP1-1607* SNP 2G allele was found to increase MMP-1 mRNA levels in healthy tissues, the strong and persistent periodontopathogens and inflammatory cytokine stimulation overcome the genetic influence and play a major role in the up-regulation of MMP-1 levels in diseased tissues. The first steps in solving the puzzle composed of genetic, inflammatory and microbial contributions to the immunopathogenesis of periodontal disease have been achieved, but further studies are required to understand their exact roles in periodontitis outcome. This knowledge might allow us to develop diagnostic, preventive and therapeutic strategies to improve the clinical management of periodontal disease.

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References

Andrian, E., Mostefaoui, Y., Rouabhia, M. & Grenier, D. (2007) Regulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases by *Porphyromonas gingivalis* in an engineered human oral mucosa model. *Journal of Cell Physiology* **211**, 56–62.

Ardans, J. A., Economou, A. P., Martinson, J. M. Jr., Zhou, M. & Wahl, L. M. (2002) Oxidized low-density and high-density lipoproteins regulate the production of matrix metalloproteinase-1 and -9 by activated monocytes. *Journal of Leukocyte Biology* **71**, 1012–1018.

Astolfi, C. M., Shinohara, A. L., da Silva, R. A., Santos, M. C., Line, S. R. & de Souza, A. P. (2006) Genetic polymorphisms in the MMP-1 and MMP-3 gene may contribute to chronic periodontitis in a Brazilian population. *Journal of Clinical Periodontology* **33**, 699–703.

Atieh, M. A. (2008) Accuracy of real-time polymerase chain reaction versus anaerobic culture in detection of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*: a meta-analysis. *Journal of Periodontology* **79**, 1620–1629.

Barksby, H. E., Nile, C. J., Jaedicke, K. M., Taylor, J. J. & Preshaw, P. M. (2009) Differential expression of immunoregulatory genes in monocytes in response to *Porphyromonas gingivalis* and *Escherichia coli* lipopolysaccharide. *Clinical and Experimental Immunology* **156**, 479–487.

Bodet, C., Chandad, F. & Grenier, D. (2006) *Porphyromonas gingivalis*-induced inflammatory mediator profile in an ex vivo human whole blood model. *Clinical and Experimental Immunology* **143**, 50–57.

Cao, Z., Li, C., Jin, L. & Corbet, E. F. (2005) Association of matrix metalloproteinase-1 promoter polymorphism with generalized aggressive periodontitis in a Chinese population. *Journal of Periodontal Research* **40**, 427–431.

Cao, Z., Li, C. & Zhu, G. (2006) MMP-1 promoter gene polymorphism and susceptibility to chronic periodontitis in a Chinese population. *Tissue Antigens* **68**, 38–43.

Chang, Y. C., Yang, S. F., Lai, C. C., Liu, J. Y. & Hsieh, Y. S. (2002) Regulation of matrix metalloproteinase production by cytokines, pharmacological agents and periodontal pathogens in human periodontal ligament fibroblast cultures. *Journal of Periodontal Research* **37**, 196–203.

Claudino, M., Trombone, A. P., Cardoso, C. R., Ferreira, S. B. Jr., Martins, W. Jr., Assis, G. F., Santos, C. F., Trevilatto, P. C., Campanelli, A. P., Silva, J. S. & Garlet, G. P. (2008) The broad effects of the functional IL-10 promoter-592 polymorphism: modulation of IL-10, TIMP-3, and OPG expression and their association with periodontal disease outcome. *Journal of Leukocyte Biology* **84**, 1565–1573.

Dall'Ozzo, S., Tartas, S., Paintaud, G., Cartron, G., Colombat, P., Bardos, P., Watier, H. & Thibault, G. (2004) Rituximab-dependent cytotoxicity by natural killer cells: influence of FCGR3A polymorphism on the concentration–effect relationship. *Cancer Research* **64**, 4664–4669.

de Souza, A. P., Trevilatto, P. C., Scarel-Caminaga, R. M., Brito, R. B. & Line, S. R. (2003) MMP-1 promoter polymorphism: association with chronic periodontitis severity in a Brazilian population. *Journal of Clinical Periodontology* **30**, 154–158.

Dean, D. D., Muniz, O. E. & Howell, D. S. (1989) Association of collagenase and tissue inhibitor of metalloproteinases (TIMP) with

hypertrophic cell enlargement in the growth plate. *Matrix* **9**, 366–375.

Ding, Y., Haapasalo, M., Kerosuo, E., Lounatmaa, K., Kotiranta, A. & Sorsa, T. (1997) Release and activation of human neutrophil matrix metallo- and serine proteinases during phagocytosis of *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Treponema denticola*. *Journal of Clinical Periodontology* **24**, 237–248.

Feng, Z. & Weinberg, A. (2006) Role of bacteria in health and disease of periodontal tissues. *Periodontology 2000* **40**, 50–76.

Ferreira, S. B. Jr., Trombone, A. P., Repeke, C. E., Cardoso, C. R., Martins, W. Jr., Santos, C. F., Trevilatto, P. C., Avila-Campos, M. J., Campanelli, A. P., Silva, J. S. & Garlet, G. P. (2008) An interleukin-1beta (IL-1beta) single-nucleotide polymorphism at position 3954 and red complex periodontopathogens independently and additively modulate the levels of IL-1beta in diseased periodontal tissues. *Infection and Immunity* **76**, 3725–3734.

Garlet, G. P., Cardoso, C. R., Silva, T. A., Ferreira, B. R., Avila-Campos, M. J., Cunha, F. Q. & Silva, J. S. (2006) Cytokine pattern determines the progression of experimental periodontal disease induced by *Actinobacillus actinomycetemcomitans* through the modulation of MMPs, RANKL, and their physiological inhibitors. *Oral Microbiology and Immunology* **21**, 12–20.

Garlet, G. P., Martins, W. Jr., Fonseca, B. A., Ferreira, B. R. & Silva, J. S. (2004) Matrix metalloproteinases, their physiological inhibitors and osteoclast factors are differentially regulated by the cytokine profile in human periodontal disease. *Journal of Clinical Periodontology* **31**, 671–679.

Godfrey, V., Chan, S. L., Cassidy, A., Butler, R., Choy, A., Fardon, T., Struthers, A. & Lang, C. (2007) The functional consequence of the Glu298Asp polymorphism of the endothelial nitric oxide synthase gene in young healthy volunteers. *Cardiovascular Drug Reviews* **25**, 280–288.

Golub, L. M., Payne, J. B., Reinhardt, R. A. & Nieman, G. (2006) Can systemic diseases co-induce (not just exacerbate) periodontitis? A hypothetical ''two-hit'' model. *Journal of Dental Research* **85**, 102–105.

Graves, D. T. & Cochran, D. (2003) The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *Journal of Periodontology* **74**, 391–401.

Hannas, A. R., Pereira, J. C., Granjeiro, J. M. & Tjaderhane, L. (2007) The role of matrix metalloproteinases in the oral environment. *Acta Odontologica Scandinavica* **65**, 1–13.

Hashimoto, R., Moriguchi, Y., Yamashita, F., Mori, T., Nemoto, K., Okada, T., Hori, H., Noguchi, H., Kunugi, H. & Ohnishi, T. (2008) Dose-dependent effect of the Val66-Met polymorphism of the brain-derived neurotrophic factor gene on memory-related hippocampal activity. *Neuroscience Research* **61**, 360–367.

Ho, H. H., Antoniv, T. T., Ji, J. D. & Ivashkiv, L. B. (2008) Lipopolysaccharide-induced expression of matrix metalloproteinases in human monocytes is suppressed by IFN-gamma via superinduction of ATF-3 and suppression of AP-1. *Journal of Immunology* **181**, 5089–5097.

Holla, L. I., Jurajda, M., Fassmann, A., Dvorakova, N., Znojil, V. & Vacha, J. (2004) Genetic variations in the matrix metalloproteinase-1 promoter and risk of susceptibility and/or severity of chronic periodontitis in the Czech population. *Journal of Clinical Periodontology* **31**, 685–690.

Hu, X. P., Xu, J. M., Hu, Y. M., Mei, Q. & Xu, X. H. (2007) Effects of CYP2C19 genetic polymorphism on the pharmacokinetics and pharmacodynamics of omeprazole in Chinese people. *Journal of Clinical Pharmacy and Therapeutics* **32**, 517–524.

Huynh-Ba, G., Lang, N. P., Tonetti, M. S. & Salvi, G. E. (2007) The association of the composite IL-1 genotype with periodontitis progression and/or treatment outcomes: a systematic review. *Journal of Clinical Periodontology* **34**, 305–317.

Itagaki, M., Kubota, T., Tai, H., Shimada, Y., Morozumi, T. & Yamazaki, K. (2004) Matrix metalloproteinase-1 and -3 gene promoter polymorphisms in Japanese patients with periodontitis. *Journal of Clinical Periodontology* **31**, 764–769.

Itoh, K., Udagawa, N., Kobayashi, K., Suda, K., Li, X., Takami, M., Okahashi, N., Nishihara, T. & Takahashi, N. (2003) Lipopolysaccharide promotes the survival of osteoclasts via Toll-like receptor 4, but cytokine production of osteoclasts in response to lipopolysaccharide is different from that of macrophages. *Journal of Immunology* **170**, 3688–3695.

Jervoe-Storm, P. M., Koltzscher, M., Falk, W., Dorfler, A. & Jepsen, S. (2005) Comparison of culture and real-time PCR for detection and quantification of five putative periodontopathogenic bacteria in subgingival plaque samples. *Journal of Clinical Periodontology* **32**, 778–783.

Kanamori, Y., Matsushima, M., Minaguchi, T., Kobayashi, K., Sagae, S., Kudo, R., Terakawa, N. & Nakamura, Y. (1999) Correlation between expression of the matrix metalloproteinase-1 gene in ovarian cancers and an insertion/deletion polymorphism in its promoter region. *Cancer Research* **59**, 4225–4227.

Kasamatsu, A., Uzawa, K., Shimada, K., Shiiba, M., Otsuka, Y., Seki, N., Abiko, Y. & Tanzawa, H. (2005) Elevation of galectin-9 as an inflammatory response in the periodontal ligament cells exposed to *Porphylomonas gingivalis* lipopolysaccharide in vitro and in vivo. *International Journal of Biochemistry and Cell Biology* **37**, 397–408.

Kiszel, P., Mako, V., Prohaszka, Z. & Cervenak, L. (2007) Interleukin-6 -174 promoter polymorphism does not influence IL-6 production after LPS and IL-1 beta stimulation in human umbilical cord vein endothelial cells. *Cytokine* **40**, 17–22.

Kou, Y., Inaba, H., Kato, T., Tagashira, M., Honma, D., Kanda, T., Ohtake, Y. & Amano, A. (2008) Inflammatory responses of gingival epithelial cells stimulated with *Porphyromonas gingivalis* vesicles are inhibited by hop-associated polyphenols. *Journal of Periodontology* **79**, 174–180.

Lai, W. C., Zhou, M., Shankavaram, U., Peng, G. & Wahl, L. M. (2003) Differential regulation of lipopolysaccharide-induced monocyte matrix metalloproteinase (MMP)-1 and MMP-9 by p38 and extracellular signal-regulated kinase 1/2 mitogen-activated protein kinases. *Journal of Immunology* **170**, 6244–6249.

Menezes, R., Garlet, T. P., Letra, A., Bramante, C. M., Campanelli, A. P., Figueira, R. C., Sogayar, M. C., Granjeiro, J. M. & Garlet, G. P. (2008) Differential patterns of receptor activator of nuclear factor kappa B ligand/osteoprotegerin expression in human periapical granulomas: possible association with progressive or stable nature of the lesions. *Journal of Endodontics* **34**, 932–938.

Mercer, B. A., Wallace, A. M., Brinckerhoff, C. E. & D'Armiento, J. M. (2009) Identification of a cigarette smoke responsive region in the distal MMP-1 promoter. *American Journal of Respiratory Cell and Molecular Biology* **40**, 4–12.

Mombelli, A. (2003) Periodontitis as an infectious disease: specific features and their implications. *Oral Diseases* **9** (Suppl. 1), 6–10.

Murphy, G., Cockett, M. I., Ward, R. V. & Docherty, A. J. (1991) Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan. A quantitative comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins-1 and -2 and punctuated metalloproteinase (PUMP). *Biochemical Journal* **277** (Part 1), 277–279.

Nawrocki, B., Polette, M., Marchand, V., Monteau, M., Gillery, P., Tournier, J. M. & Birembaut, P. (1997) Expression of matrix metalloproteinases and their inhibitors in human bronchopulmonary carcinomas: quantificative and morphological analyses. *International Journal of Cancer* **72**, 556–564.

Nishikawa, M., Yamaguchi, Y., Yoshitake, K. & Saeki, Y. (2002) Effects of TNFalpha and prostaglandin E2 on the expression of MMPs in human periodontal ligament fibroblasts. *Journal of Periodontal Research* **37**, 167–176.

Nonnenmacher, C., Dalpke, A., Mutters, R. & Heeg, K. (2004) Quantitative detection of periodontopathogens by real-time PCR. *Journal of Microbiological Methods* **59**, 117–125.

Parra, F. C., Amado, R. C., Lambertucci, J. R., Rocha, J., Antunes, C. M. & Pena, S. D. (2003) Color and genomic ancestry in Brazilians. *Proceedings of National Academy of Sciences USA* **100**, 177–182.

Pirhan, D., Atilla, G., Emingil, G., Sorsa, T., Tervahartiala, T. & Berdeli, A. (2008) Effect of MMP-1 promoter polymorphisms on GCF MMP-1 levels and outcome of periodontal therapy in patients with severe chronic periodontitis. *Journal of Clinical Periodontology* **35**, 862–870.

Pirmohamed, M. & Park, B. K. (2001) Genetic susceptibility to adverse drug reactions. *Trends in Pharmacological Sciences* **22**, 298–305.

Pussinen, P. J., Paju, S., Mantyla, P. & Sorsa, T. (2007) Serum microbial- and host-derived

markers of periodontal diseases: a review. *Current Medicinal Chemistry* **14**, 2402–2412.
Pussinen, P. J., Vilkuna-Rautiainen, T., Alfthan, G., Palosuo, T., Jauhiainen, M., Sundvall, J., Vesanen, M., Mattila, K. & Asikainen, S. (2004) Severe periodontitis enhances macrophage activation via increased serum lipopolysaccharide. *Arteriosclerosis, Thrombosis, and Vascular Biology* **24**, 2174–2180.
Reynolds, J. J. (1996) Collagenases and tissue inhibitors of metalloproteinases: a functional balance in tissue degradation. *Oral Diseases* **2**, 70–76.
Reynolds, J. J., Hembry, R. M. & Meikle, M. C. (1994) Connective tissue degradation in health and periodontal disease and the roles of matrix metalloproteinases and their natural inhibitors. *Advances in Dental Research* **8**, 312–319.
Rossa, C. Jr., Liu, M., Bronson, P. & Kirkwood, K. L. (2007) Transcriptional activation of MMP-13 by periodontal pathogenic LPS requires p38 MAP kinase. *Journal of Endotoxin Research* **13**, 85–93.
Sanz, M., Lau, L., Herrera, D., Morillo, J. M. & Silva, A. (2004) Methods of detection of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythensis* in periodontal microbiology, with special emphasis on advanced molecular techniques: a review. *Journal of Clinical Periodontology* **31**, 1034–1047.
Shah, R. R. (2005) Mechanistic basis of adverse drug reactions: the perils of inappropriate dose schedules. *Expert Opinion on Drug Safety* **4**, 103–128.
Shapira, L., Wilensky, A. & Kinane, D. F. (2005) Effect of genetic variability on the inflammatory response to periodontal infection. *Journal of Clinical Periodontology* **32** (Suppl. 6), 72–86.
Sternlicht, M. D. & Werb, Z. (2001) How matrix metalloproteinases regulate cell behavior. *Annual Review of Cell and Developmental Biology* **17**, 463–516.
Sundararaj, K. P., Samuvel, D. J., Li, Y., Nareika, A., Slate, E. H., Sanders, J. J., Lopes-Virella, M. F. & Huang, Y. (2008) Simvastatin suppresses LPS-induced MMP-1 expression in U937 mononuclear cells by inhibiting protein isoprenylation-mediated ERK activation. *Journal of Leukocyte Biology* **84**, 1120–1129.
Tai, E. S., Corella, D., Demissie, S., Cupples, L. A., Coltell, O., Schaefer, E. J., Tucker, K. L. & Ordovas, J. M. (2005) Polyunsaturated fatty acids interact with the PPARA-L162V polymorphism to affect plasma triglyceride and apolipoprotein C-III concentrations in the Framingham Heart Study. *Journal of Nutrition* **135**, 397–403.
Tiranathanagul, S., Yongchaitrakul, T., Pattamapun, K. & Pavasant, P. (2004) *Actinobacillus actinomycetemcomitans* lipopolysaccharide activates matrix metalloproteinase-2 and increases receptor activator of nuclear factor-kappaB ligand expression in human periodontal ligament cells. *Journal of Periodontology* **75**, 1647–1654.
Tonetti, M. S. & Claffey, N. (2005) Advances in the progression of periodontitis and proposal of definitions of a periodontitis case and disease progression for use in risk factor research. Group C consensus report of the 5th European Workshop in Periodontology. *Journal of Clinical Periodontology* **32** (Suppl. 6), 210–213.
Trevilatto, P. C. & Line, S. R. (2000) Use of buccal epithelial cells for PCR amplification of large DNA fragments. *Journal of Forensic Odonto-Stomatology* **18**, 6–9.
Trombone, A. P., Cardoso, C. R., Repeke, C. E., Ferreira, S. B. Jr., Martins, W. Jr., Campanelli, A. P., Avila-Campos, M. J., Trevilatto, P. C., Silva, J. S. & Garlet, G. P. (2008) Tumor necrosis factor-alpha-308G/A single nucleotide polymorphism and red-complex periodontopathogens are independently associated with increased levels of tumor necrosis factor-alpha in diseased periodontal tissues. *Journal of Periodontal Research*.
Ustun, K., Alptekin, N. O., Hakki, S. S. & Hakki, E. E. (2008) Investigation of matrix metalloproteinase-1–1607 1G/2G polymorphism in a Turkish population with periodontitis. *Journal of Clinical Periodontology* **35**, 1013–1019.
van der Zee, E., Everts, V. & Beertsen, W. (1997) Cytokines modulate routes of collagen breakdown. Review with special emphasis on mechanisms of collagen degradation in the periodontium and the burst hypothesis of periodontal disease progression. *Journal of Clinical Periodontology* **24**, 297–305.
Verstappen, J. & Von den Hoff, J. W. (2006) Tissue inhibitors of metalloproteinases (TIMPs): their biological functions and involvement in oral disease. *Journal of Dental Research* **85**, 1074–1084.
Wilson, M. E. & Hamilton, R. G. (1992) Immunoglobulin G subclass response of localized juvenile periodontitis patients to *Actinobacillus actinomycetemcomitans* Y4 lipopolysaccharide. *Infection and Immunity* **60**, 1806–1812.
Ximénez-Fyvie, L. A., Haffajee, A. D. & Socransky, S. S. (2000) Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis. *Journal of Clinical Periodontology* **27**, 722–732.
Yamaji, Y., Kubota, T., Sasaguri, K., Sato, S., Suzuki, Y., Kumada, H. & Umemoto, T. (1995) Inflammatory cytokine gene expression in human periodontal ligament fibroblasts stimulated with bacterial lipopolysaccharides. *Infection and Immunity* **63**, 3576–3581.
Ye, S. (2000) Polymorphism in matrix metalloproteinase gene promoters: implication in regulation of gene expression and susceptibility of various diseases. *Matrix Biology* **19**, 623–629.
Zhou, J. & Windsor, L. J. (2006) *Porphyromonas gingivalis* affects host collagen degradation by affecting expression, activation, and inhibition of matrix metalloproteinases. *Journal of Periodontal Research* **41**, 47–54.
Zhou, J. & Windsor, L. J. (2007) Heterogeneity in the collagen-degrading ability of *Porphyromonas gingivalis*-stimulated human gingival fibroblasts. *Journal of Periodontal Research* **42**, 77–84.

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Clinical Relevance

Scientific rationale for the study: Host genetic background, specific periodontopathogens and cytokines may determine individual variations in MMP-1 levels and periodontitis outcome. Therefore, the simultaneous investigation of these factors may unravel its individual role in the modulation of MMP-1 mRNA expression and therefore provide important information in periodontal disease.

Principal findings: No significant differences were found in the frequency of *MMP1-1607* genotypes in the C and CP groups. In healthy tissues, the *MMP1-1607* 2G allele was associated with higher MMP-1 levels, while red complex periodontopathogens and inflammatory cytokines TNF-α and IL-1β were positively correlated with increased MMP-1 expression in diseased periodontium of non-smokers. The *MMP1-1607* 2G allele influences MMP-1 expression in vitro only in response to low-degree microbial and inflammatory stimulation, while strong and repeated stimulation resulted in higher MMP-1 levels irrespective of the *MMP1-1607* genotype.

Practical implications: *MMP1-1607* SNP was not a useful marker to assess the susceptibility to periodontal disease. On the other hand, our results demonstrate that the chronic antigenic challenge exposure in periodontal disease may overcome the genetic influence and plays a major role in periodontal disease outcome.