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Tumor necrosis factor-alpha −308G/A single nucleotide polymorphism and red-complex periodontopathogens are independently associated with increased levels of tumor necrosis factor-α in diseased periodontal tissues

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Background and Objective: Inflammatory cytokines such as tumor necrosis factor-alpha are involved in the pathogenesis of periodontal diseases. A high between-subject variation in the level of tumor necrosis factor-alpha mRNA has been verified, which may be a result of genetic polymorphisms and/or the presence of periodontopathogens such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* (called the red complex) and *Aggregatibacter actinomycetemcomitans*. In this study, we investigated the effect of the tumor necrosis factor-alpha (*TNFA*) −308G/A gene polymorphism and of periodontopathogens on the tumor necrosis factor-alpha levels in the periodontal tissues of nonsmoking patients with chronic periodontitis ($n = 127$) and in control subjects ($n = 177$).

Material and Methods: The *TNFA* −308G/A single nucleotide polymorphism was investigated using polymerase chain reaction–restriction fragment length polymorphism analysis, whereas the tumor necrosis factor-alpha levels and the periodontopathogen load were determined using real-time polymerase chain reaction.

Results: No statistically significant differences were found in the frequency of the *TNFA* −308 single nucleotide polymorphism in control and chronic periodontitis groups, in spite of the higher frequency of the A allele in the chronic periodontitis

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group. The concomitant analyses of genotypes and periodontopathogens demonstrated that *TNFA* −308 GA/AA genotypes and the red-complex periodontopathogens were independently associated with increased levels of tumor necrosis factor-alpha in periodontal tissues, and no additive effect was seen when both factors were present. *P. gingivalis*, *T. forsythia* and *T. denticola* counts were positively correlated with the level of tumor necrosis factor-alpha. *TNFA* −308 genotypes were not associated with the periodontopathogen detection odds or with the bacterial load.

Conclusion: Our results demonstrate that the *TNFA* −308 A allele and red-complex periodontopathogens are independently associated with increased levels of tumor necrosis factor-alpha in diseased tissues of nonsmoking chronic periodontitis patients and consequently are potentially involved in determining the disease outcome.

Periodontal diseases are infectious diseases in which periodontopathogens trigger chronic inflammatory and immune responses that are thought to determine the clinical outcome of the disease (1). The presence of periodontopathogens, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* (called the red complex) and *Aggregatibacter actinomycetemcomitans*, which are considered to be the major etiologic agents of periodontitis (2,3), triggers the expression of pro-inflammatory cytokines such as tumor necrosis factor-alpha that have been associated with the immunopathology of periodontitis (4). In fact, tumor necrosis factor-alpha is present at high levels in both gingival crevicular fluid and diseased periodontal tissues, and experimental studies have demonstrated a central role for tumor necrosis factor-alpha in the inflammatory reaction and in alveolar bone resorption (4,5).

Interestingly, variation in cytokine levels among patients with periodontitis is well documented and is associated with disease susceptibility (6,7). Heritable differences are reported in tumor necrosis factor-alpha production, and single nucleotide polymorphisms, particularly the G to A transition at position −308, have been demonstrated to affect the binding of transcription factors responsible for increasing tumor necrosis factor-alpha production up to fivefold *in vitro* and have also been associated with increased cytokine levels *in vivo* (8,9). Therefore, the tumor necrosis factor-alpha (*TNFA*) −308 A allele has been associated with increased risk for the development of diverse infectious and inflammatory diseases (10–12). Indeed, the *TNFA* −308 A allele was demonstrated to be associated with higher levels of tumor necrosis factor-alpha production by oral polymorphonuclear neutrophils from patients with adult periodontitis (13) and with the severity of chronic periodontitis (14).

In addition to the influence of the host genetic background, specific periodontopathogens may also account for higher tumor necrosis factor-alpha levels in periodontal tissues. Indeed, the red-complex periodontopathogens and *A. actinomycetemcomitans* characteristically induce tumor necrosis factor-alpha production (15,16). However, the putative influences of the periodontopathogens and of the *TNFA* −308 single nucleotide polymorphism have been investigated independently, and consequently their exact individual and/or combined contribution in determining the tumor necrosis factor-alpha levels in diseased periodontal tissues remains unknown. Therefore, the aim of this study was to evaluate the role of the *TNFA* −308 single nucleotide polymorphism and of *P. gingivalis*, *T. forsythia*, *T. denticola* and *A. actinomycetemcomitans*, in the modulation of the tumor necrosis factor-alpha mRNA levels in the periodontal tissues of patients with chronic periodontitis.

Material and methods

Study population and clinical examination

Patients and controls, from the southeastern region of Brazil, scheduled for treatment at the Dentistry School of University of Ribeirão Preto (UNAERP), were submitted to anamnesis and to clinical (scored for bleeding on probing, probing depth and clinical attachment loss) and radiographic examinations, and then were categorized into control or chronic periodontitis groups, as previously described (17,18). Prior to starting the study, all subjects signed a consent form that was approved by an Institutional Review Board, and received supragingival prophylaxis. Exclusion criteria were as follows: not providing informed consent; a significant medical history indicating evidence of known systemic modifiers of periodontal disease; or undergoing periodontal therapy in the previous 2 years, as previously described (17). Smokers were specifically excluded.

After the diagnostic phase, patients in the chronic periodontitis group ($n = 127$), presenting moderate to advanced probing depth (at least one teeth per sextant with probing depth > 6 mm and clinical attachment loss > 3 mm), received basic periodontal therapy. Biopsies of gingival tissue (one sample from each patient) were obtained during surgical therapy of the sites that exhibited no

improvement in clinical condition (i.e. persistent bleeding on probing and higher probing depth) 3–4 wk after the basic periodontal therapy, as previously described (17). The control group ($n = 177$) comprised subjects with clinically healthy gingival tissues (< 10% of bleeding on probing; no sites with probing depth > 3 mm or presenting clinical attachment loss) scheduled to undergo restorative dentistry procedures. A representative fraction of the control group ($n = 63$; 30 women/33 men; age 42.82 ± 7.58 years; genotypes: 45GG, 11GA, 7AA) was also scheduled to undergo surgical procedures for restorative/prosthetic reasons, when biopsies of healthy gingival tissue (no bleeding on probing and probing depth < 3 mm) were taken. The clinical features of the groups are summarized in Table 1.

Analysis of genetic polymorphisms

DNA was extracted from epithelial buccal cells using sequential phenol and chlorophorm extraction procedures and precipitated with salt/ethanol solution (6.0 M GuSCN, 65 mM Tris-HCL, with 450 μL 70% ethanol) (19). For genotyping *TNFA* −308, DNA fragments were amplified by use of the following primer pair: 5′-AGGCAATAGGTTTTGAGGGCCAT-3′ and 5′-TCCTCCCTGCTCCGATTCCG-3′ (19). The polymerase chain reaction (PCR) was performed in a 25-μL reaction mixture containing PCR buffer (Promega Corporation, Madison, Wisconsin, USA), 2.5 mM $MgCl_2$, 10 mM dNTPs, 100 nM each primer, 200 ng of sample DNA and 2 U Taq polymerase. The PCR cycle conditions were 95°C for 5 min, followed by 35 cycles at 95°C for 45 s, 60°C for 45 s, 72°C for 45 s and 72°C for 10 min. A 15-μL aliquot of PCR product was subjected to restriction fragment length polymorphism with 3 U of *Nco*I (Promega) at 37°C overnight, and the products were resolved and separated on a 2% agarose gel stained with SYBRsafe (Invitrogen Life Technologies, Carlsbad, CA, USA), in order to yield the genotypes AA (107 bp), GA (107 + 87 + 20 bp) and GG (80 + 20 bp).

Real-time PCR reactions

The extraction of total RNA from periodontal tissue samples using Trizol reagent (Invitrogen), and cDNA synthesis, were accomplished as previously described (5). For detection of *P. gingivalis*, *T. forsythia*, *T. denticola* and *A. actinomycetemcomitans*, periodontal crevice/pocket biofilm samples were collected, from the same site biopsied previously to the surgical procedure, using a sterile paper point ISO #40 (Tanari Industrial Ltda, São Paulo, Brazil) (20). Bacterial DNA was extracted from plaque samples using a DNA-purification system (Promega). Real-time PCR mRNA or DNA analyses were performed in a MiniOpticon system (Bio-Rad, Hercules, CA, USA), using SybrGreen MasterMix (Invitrogen), specific primers (17,21,22) and 2.5 ng of cDNA or 5 ng of DNA in each reaction. For mRNA analysis, the relative level of gene expression was calculated in reference to beta-actin using the cycle threshold method. The presence of bacteria and the bacterial counts in each sample were determined based on comparison with a standard curve produced using specific bacterial DNA (from 10^9 to 10^{-2} bacteria) and negative controls, similar to that described previously (20), and then adjusted for sample dilution in the assay to give the bacterial copy numbers in each sample. The sensitivity range of

Table 1. Clinical features and frequencies of the tumor necrosis factor-alpha (*TNFA*) −308 single nucleotide polymorphism in control (C) subjects and in chronic periodontitis (CP) patients

	C ($n = 177$)	CP ($n = 127$)	*p*-value
Gender	83F/94M	59F/68M	NS*
Age	42.3 ± 7.61	46.7 ± 7.40	NS**
Ethnic group, *n* (%)			
Caucasoid	150 (84)	106 (82.68)	$p = 0.7531$, OR = 1.101, 95% CI = 0.5907 to 2.051
Afro-American/Mulatto	27 (16)	21 (16.38)	
Clinical parameters, value ± SD			
Probing depth (mean)	2.17 ± 0.65	4.27 ± 0.73	< 0.0001**
Probing depth (site)	2.24 ± 0.54	7.05 ± 1.16	< 0.0001**
Attachment loss (site)	0	3.94 ± 1.12	Nd
% BOP (mean)	5.18 ± 1.63	62.58 ± 11.71	< 0.0001**
TNFA −308 genotypes (*n*/%)			*p*-value*
GG	142/79.52	92/71.76	
GA	24/13.44	25/19.5	$p = 0.1517$, OR = 1.608, 95% CI = 0.8661–2.985
AA	11/6.16	10/7.8	$p = 0.4911$, OR = 1.403, 95% CI = 0.5728–3.437
GA + AA	35/19.6	35/27.3	$p = 0.1291$, OR = 1.543, 95% CI = 0.9021–2.641
TNFA −308 alleles (*n*/%)			
G	308/87	209/82.28	$p = 0.1337$, OR = 1.442, 95% CI = 0.9220–2.254
A	46/13	45/17.72	

BOP, bleeding on probing; CI, confidence interval; F, female; M, male; ND, not determined; NS, not significant; OR, odds ratio.
*Fisher's Exact Test.
**Unpaired *t*-test.

bacterial detection and quantification of the real-time PCR technique used was 10^1–10^8 bacteria for each of the four periodontopathogens tested.

Statistical analysis

The significance of the differences in observed frequencies of the polymorphism studied was assessed using Fisher's Exact Test, and the risk associated with genotypes/alleles was calculated as the odds ratio with 95% confidence intervals. Analysis of possible differences between the genotypes subgroups of control and chronic periodontitis groups was carried out using analysis of variance, followed by Tukey's test, while multiple logistic and linear regression analyses were performed to evaluate possible associations between the variables. Values of $p < 0.05$ were considered to be statistically significant.

Results

TNFA −308G/A single nucleotide polymorphism frequency analysis in control and chronic periodontitis groups

The subject sample included in this study comprised a similar number of male and female subjects (Table 1), and the distribution of genotypes was found to be in Hardy–Weinberg

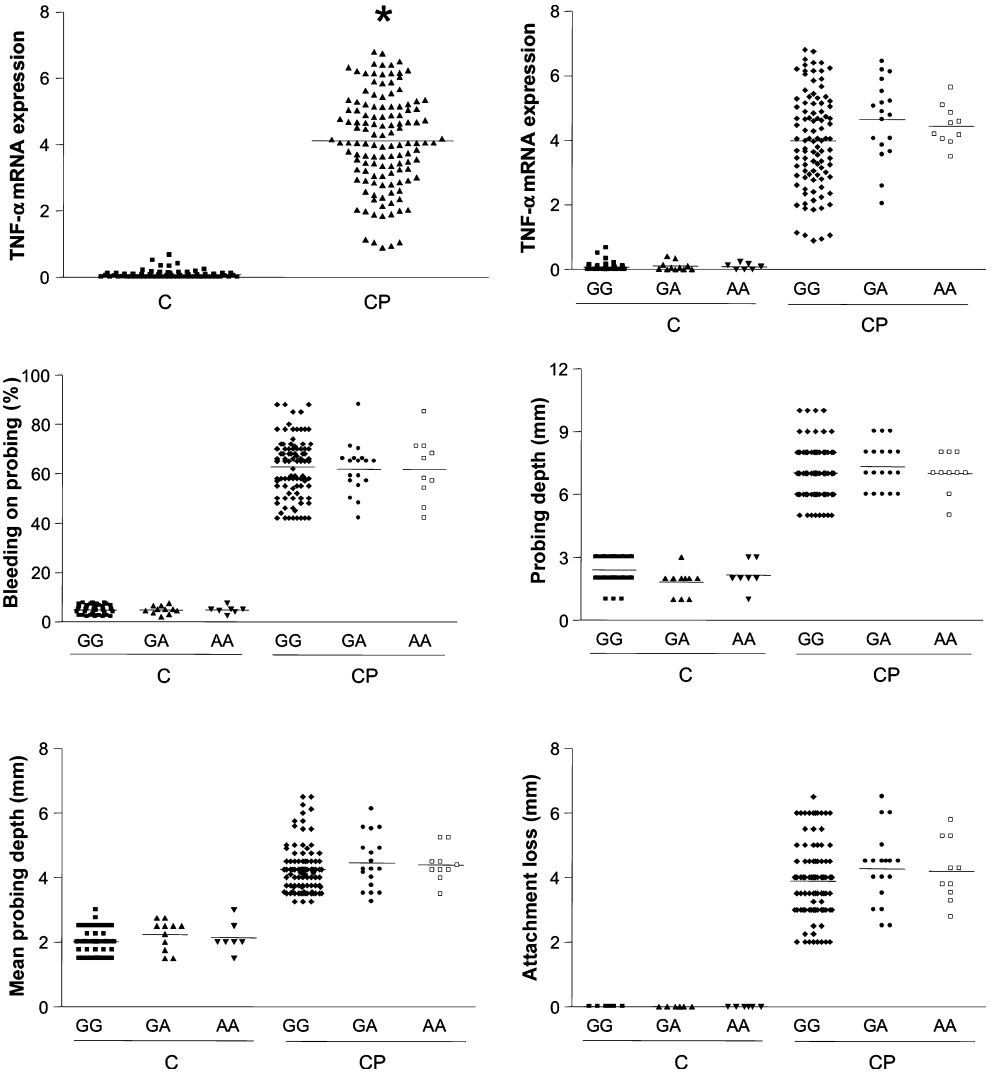

Fig. 1. Association of the tumor necrosis factor-alpha (*TNFA*) −308 single nucleotide polymorphism genotypes with tumor necrosis factor-alpha expression and clinical parameters of periodontitis severity. Control subjects and chronic periodontitis patients were subjected to periodontal examination, and the genotype of the *TNFA* −308 single nucleotide polymorphism was determined using restriction fragment length polymorphism analysis. Total RNA was extracted from gingival tissues, and the levels of tumor necrosis factor-alpha mRNA were measured quantitatively using real-time polymerase chain reaction. The results are presented as expression of the individual mRNAs, with normalization to beta-actin. The graphs depict the expression of tumor necrosis factor-alpha and the clinical parameters of disease severity (bleeding on probing, probing depth, mean probing depth, attachment loss) in control subjects and in chronic periodontitis patients regarding the *TNFA* −308 single nucleotide polymorphism genotype. All genotype subgroups of chronic periodontitis patients were significantly different from those of the control group. C, control; CP, chronic periodontitis; TNF-α, tumor necrosis factor-alpha.
*$P < 0.001$, Student's *t*-test.

equilibrium. Caucasians were prevalent compared with Afro-Americans/Mulatto individuals; however, no further analysis was performed based on such classification in view of the high genetic miscegenation of the Brazilian population (23). The frequency of the *TNFA* −308 polymorphic genotypes GA and AA, and of the allele A, were found to be similar in chronic periodontitis and control groups (Table 1), and were also similar to that reported for the Brazilian population (19,24,25).

Genotype vs. tumor necrosis factor-alpha levels and clinical parameters

To evaluate the putative functionality of the *TNFA* −308 single nucleotide polymorphism, we correlated it with the expression of tumor necrosis factor-alpha mRNA in periodontal tissues (Fig. 1). Our data show the absence of, or very weak, expression of tumor necrosis factor-alpha in periodontal tissues of control subjects, whereas a significantly stronger expression of tumor necrosis factor-alpha was found in the chronic periodontitis group. No differences were found between the tumor necrosis factor-alpha levels and clinical parameters in the different genotypes of the control group, whereas GA and AA carriers from the chronic periodontitis group presented a small, statistically insignificant, increase in the mean tumor necrosis factor-alpha level when compared with carriers of the GG genotype (Fig. 1). Similar clinical scores of bleeding on probing and probing depth were seen in all genotype groups (Fig. 1). We found that tumor necrosis factor-alpha levels were positively correlated with the levels of attachment loss ($r^2 = 0.08204$, $p = 0.0011$), probing depth ($r^2 = 0.1212$, $p < 0.0001$) and mean probing depth ($r^2 = 0.1933$, $p = 0.0017$).

Periodontopathogens vs. tumor necrosis factor-alpha levels and clinical parameters

The periodontopathogens were found to be significantly more prevalent in patients of the chronic periodontitis group than in patients of the control group (Table 2), and the *P. gingivalis*, *T. forsythia, T. denticola* and *A. actinomycetemcomitans* counts were found to be notably higher in chronic periodontitis patients (Fig. 2). In the

Table 2. Frequencies of periodontopathogen species in control (C) subjects and in chronic periodontitis (CP) patients regarding their tumor necrosis factor-alpha (*TNFA*) −308 genotype

	C ($n = 63$)		CP ($n = 117$)		
Bacteria	Negative (n/%)	Positive (n/%)	Negative (n/%)	Positive (n/%)	Fisher's Exact Test
Porphyromonas gingivalis	**54 (85.32)**	**9 (14.22)**	**49 (38.22)**	**78 (60.84)**	**$p < 0.0001$, OR = 9.551, 95% CI: 4.330–21.069**
GG	40 (63.20)	5 (7.90)	36 (28.08)	63 (49.14)	
GA + AA	14 (22.12)	4 (6.32)	13 (12.22)	15 (11.70)	
	$p = 0.2619$, OR = 2.286, 95% CI 0.5366–9.736		$p = 0.3823$, OR = 0.6593, 95% CI 0.2823–1.540		
Treponema denticola	**56 (88.48)**	**7 (11.06)**	**54 (42.12)**	**73 (56.94)**	**$p < 0.0001$, OR = 10.815, 95% CI: 4.571–25.585**
GG	41 (64.78)	4 (6.32)	41 (31.98)	58 (45.24)	
GA + AA	15 (23.25)	3 (4.74)	13 (12.22)	15 (11.70)	
	$p = 0.3971$, OR = 2.050, 95% CI: 0.4098–10.255		$p = 0.6694$, OR = 0.8156, 95% CI: 0.3508–1.896		
Tannerella forsythia	**58 (91.64)**	**5 (7.90)**	**53 (41.34)**	**74 (57.72)**	**$p < 0.0001$, OR = 16.196, 95% CI: 6.082–43.132**
GG	43 (67.94)	2 (3.16)	39 (30.42)	60 (46.80)	
GA + AA	15 (23.70)	3 (4.74)	14 (10.92)	14 (10.92)	
	$p = 0.1357$, OR = 4.300, 95% CI: 0.6537–28.285		$p = 0.3865$, OR = 0.6500, 95% CI: 0.2796–1.511		
Red complex	**45 (71.10)**	**18 (28.44)**	**29 (22.62)**	**98 (76.44)**	**$p < 0.0001$, OR = 8.448, 95% CI: 4.254–16.777**
GG	35 (55.30)	10 (15.80)	21 (16.38)	78 (60.84)	
GA + AA	10 (15.80)	8 (12.64)	8 (6.24)	20 (15.60)	
	$p = 0.1214$, OR = 2.800, 95% CI: 0.8728–8.983		$p = 0.4482$, OR = 0.6731, 95% CI: 0.2600–1.743		
Aggregatibacter actinomycetemcomitans	**61 (96.38)**	**2 (3.16)**	**106 (82.68)**	**21 (16.38)**	**$p = 0.0082$, OR = 6.042, 95% CI: 1.369–26.666**
GG	44 (69.52)	1 (1.58)	81 (63.18)	17 (13.26)	
GA + AA	17 (22.12)	1 (1.58)	25 (19.50)	4 (3.12)	
	$p = 0.4931$, OR = 2.588, 95% CI: 0.1530–43.791		$p = 0.7812$, OR = 0.7624, 95% CI: 0.2347–2.476		

Fisher's Exact Test was performed to determine the odds of detecting periodontopathogens in control subjects and chronic periodontitis patients and also in the different genotype subgroups (CC vs. CT + TT); subjects were considered red-complex positive when at least one of the red-complex species was detected.

The values of Fischer's exact test in the right column are derived from analysis of the values of positive and negative subjects to each bacteria (or red complex) detection in C versus CP groups, and these values were styled in bold to highlight such association and facilitate the visualization of the table.

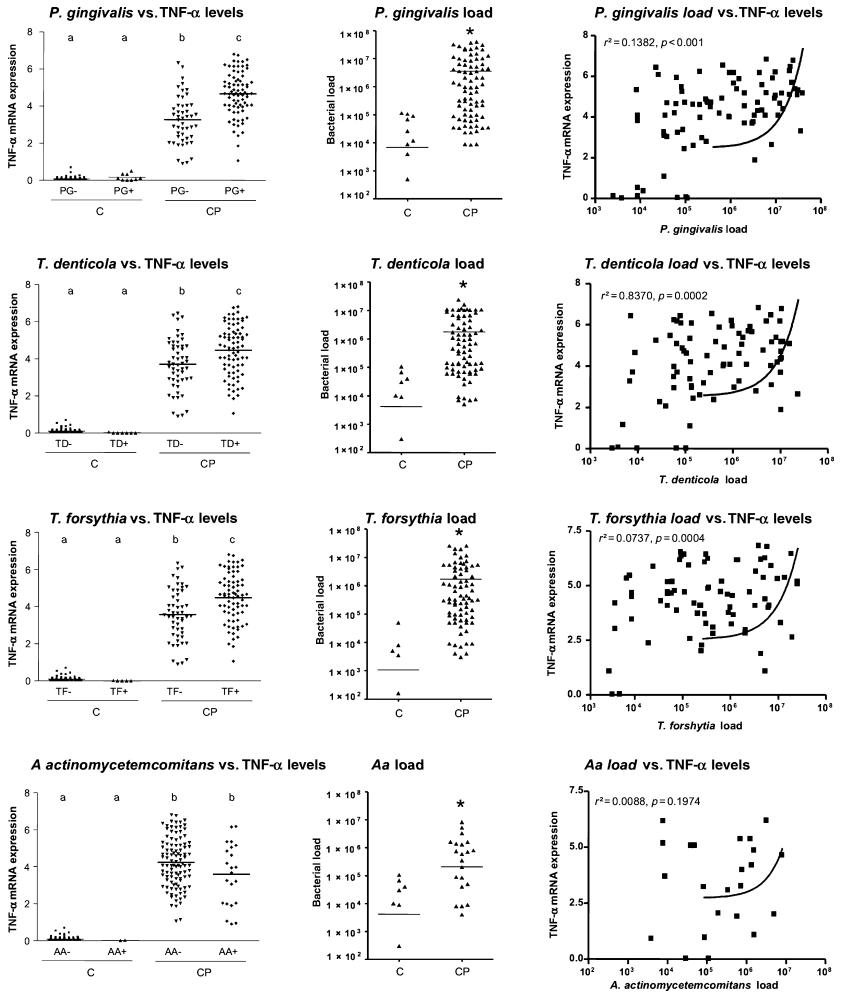

Fig. 2. Association between the presence and load of periodontopathogens and the expression of tumor necrosis factor-alpha mRNA. Control subjects and chronic periodontitis patients were subjected to periodontal examination, and the genotype of the tumor necrosis factor-alpha (*TNFA*) −308 single nucleotide polymorphism was determined using restriction fragment length polymorphism analysis. Total RNA was extracted from gingival tissues, and the levels of tumor necrosis factor-alpha mRNA were measured quantitatively using real-time polymerase chain reaction. The results are presented as expression of the individual mRNAs, with normalization to beta-actin. The presence and load of the periodontopathogens *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans* were determined by real-time polymerase chain reaction. The graphs depict the expression of tumor necrosis factor-alpha in control subjects and in chronic periodontitis patients regarding their positivity for the presence of each species of bacteria. ($p < 0.05$, analysis of variance: different letters indicate statistical significance); the bacterial load in each group (*$p < 0.05$, Student's *t*-test), and the association between the periodontopathogen load and tumor necrosis factor-alpha mRNA levels (values of r^2 and the *p*-value of linear regression analysis are indicated in the graphs). *Aa*, *Aggregatibacter actinomycetemcomitans*; C, control; CP, chronic periodontitis; TNF-α, tumor necrosis factor-alpha.

chronic periodontitis group, detection of periodontopathogens of the red complex was associated with significantly higher expression of tumor necrosis factor-alpha mRNA, and the loads of *P. gingivalis* ($r^2 = 0.1382$, $p < 0.001$), *T. forsythia* ($r^2 = 0.0737$, $p = 0.0004$) and *T. denticola* ($r^2 = 0.8370$, $p = 0.0002$) were also positively correlated with tumor necrosis factor-alpha mRNA expression (Fig. 2). Our data demonstrate a high prevalence of the simultaneous occurrence of red-complex periodontopathogens in chronic periodontitis patients ($n = 78$/60.84% presented at least two red-complex species). Additionally, we found that the presence of only one species of bacteria of the red complex did not result in increased levels of tumor necrosis factor-alpha mRNA, whereas the presence of two or three red-complex species was found to be associated with a higher expression of tumor necrosis factor-alpha in diseased tissues (Fig. 4). Regarding the possible association between the clinical parameters and the presence of the red-complex periodontopathogens, *P. gingivalis* detection was associated with higher attachment loss, probing depth and mean probing depth, whereas *T. forsythia* and *T. denticola* were found to be associated with higher values of probing depth (data not shown), similar to that reported in previous studies (2,18,26).

Genotype and periodontopathogens vs. tumor necrosis factor-alpha mRNA expression

To investigate the individual roles of the *TNFA* −308 genotype and periodontopathogens in determining tumor necrosis factor-alpha levels in diseased periodontium, patients of the chronic periodontitis group were clustered regarding the TNF genotype concurrently to the absence/presence of each target bacteria (Fig. 3). Our results demonstrate that in the absence of *P. gingivalis*, *T. forsythia* or *T. denticola*, the presence of the allele A (GA + AA groups) was associated with higher levels of tumor necrosis factor-alpha. However, in the presence of red-complex periodontopathogens all *TNFA* −308 genotype groups presented a similarly high level of tumor necrosis factor-alpha expression. Interestingly, red-complex bacteria-negative subjects bearing GA/AA genotypes presented levels of tumor necrosis factor-alpha that were similar to those of the red-complex bacteria-positive patients irrespective of their genotypes. Similarly, when the red-complex species were evaluated as a group, we found that the occurrence of the A allele was associated with higher levels of tumor

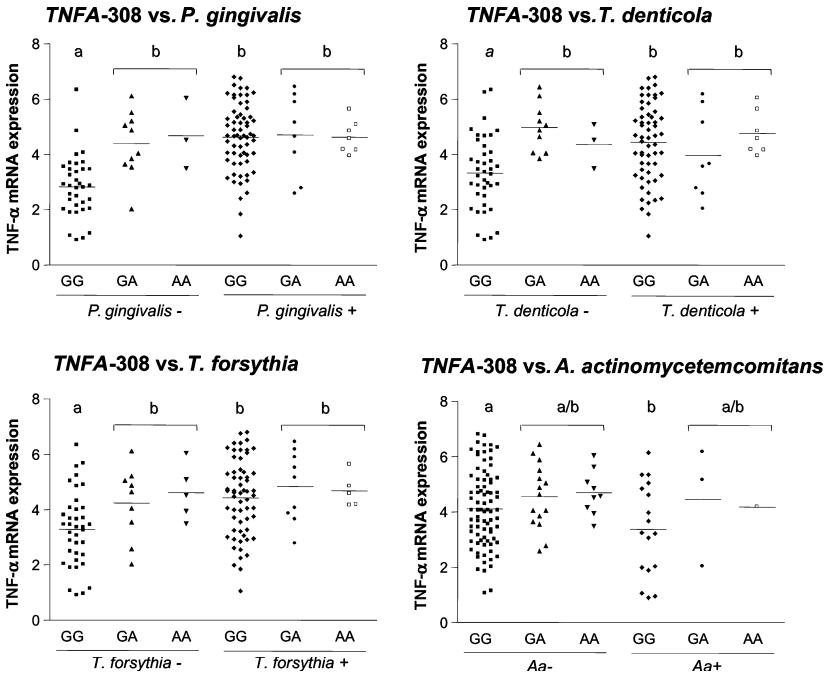

Fig. 3. Quantitative assessment of tumor necrosis factor-alpha mRNA expression in the presence or absence of periodontopathogens associated with the genotypes in the chronic periodontitis group. Control subjects and chronic periodontitis patients were subjected to periodontal examination, and the genotype of the tumor necrosis factor-alpha (*TNFA*) −308 single nucleotide polymorphism was determined by restriction fragment length polymorphism analysis. Total RNA was extracted from gingival tissues, and the levels of tumor necrosis factor-alpha mRNA were measured quantitatively using real-time polymerase chain reaction. The results are presented as expression of the individual mRNAs, with normalization to beta-actin. The presence of the periodontopathogens *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans* was investigated by real-time polymerase chain reaction. The graphs depict the expression of tumor necrosis factor-alpha in chronic periodontitis patients regarding their *TNFA* −308 single nucleotide polymorphism genotype concomitantly with their positivity for the presence of each species of periodontopathogen; $p < 0.05$ (analysis of variance): different letters indicate statistical significance. *Aa*, *Aggregatibacter actinomycetemcomitans*; C, control; CP, chronic periodontitis; TNF-alpha, tumor necrosis factor-alpha.

necrosis factor-alpha in red-complex bacteria-negative subjects, whereas in the presence of red-complex bacteria no differences were found in the levels of tumor necrosis factor-alpha in the different genotype carriers (Fig. 4). Multiple logistic regression analysis demonstrated that tumor necrosis factor-alpha expression is age ($p = 0.447$, odds ratio = 0.9, confidence interval = 0.9–1.1), race ($p = 0.498$, odds ratio = 1.0, confidence interval = 0.4–2.4) and gender ($p = 0.749$, odds ratio = 1.2, confidence interval = 0.5–2.8) independent, being only associated with the red-complex periodontopathogens ($p = 0.009$, odds ratio = 3.6, confidence interval = 1.2–8.2) and with the *TNFA* −308 genotype ($p = 0.011$, odds ratio = 3.3, confidence interval = 1.0–6.8). *A. actinomycetemcomitans* was not found to be associated with changes in the levels of tumor necrosis factor-alpha. No differences were found in the frequency or in the load of the periodontopathogens investigated in the different *TNFA* −308 genotype groups (Table 2 and Fig. 5).

Discussion

Periodontal diseases are chronic inflammatory infectious diseases that lead to the destruction of the tooth-supporting apparatus. While the presence of periodontopathogens (such as *P. gingivalis*, *T. forsythia*, *T. denticola* and *A. actinomycetemcomitans*) is an essential requirement for the development of disease (3,27), the nature of the host response raised against these bacteria strongly influences disease outcome (1). The levels of the pro-inflammatory cytokine tumor necrosis factor-alpha are characteristically increased in diseased periodontium, where it is a critical determinant of tissue destruction as a result of its pro-inflammatory and bone-resorptive properties (4,17,28). In accordance, our data demonstrate a significantly higher expression of tumor necrosis factor-alpha in the tissues of chronic periodontitis patients when compared with controls. Interestingly, the presence of a single nucleotide polymorphism in the −308 position of the *TNFA* gene has been associated with increased tumor necrosis factor-alpha synthesis by oral polymorphonuclear neutrophils from periodontitis patients (13), and also with the disease severity (14). The results presented here demonstrate similar frequencies of the *TNFA* −308 single nucleotide polymorphism genotypes in patients with chronic periodontitis and in control subjects, in spite of a trend towards a higher frequency of A-allele occurrence in the disease group. Indeed, the frequency of the *TNFA* −308 polymorphism has not been found to be associated with periodontal diseases (29–33). However, genetic studies in complex-trait multifactorial diseases such as periodontitis are complicated, and the observance (or not) of covariates (smoking and specific bacteria) and the criteria for selecting diseased and control groups may hinder their interpretation (7,34). In fact, in such studies the control group comprises periodontally healthy subjects, which does not necessarily imply a genetic resistance to the disease development but may only reflect the control of the etiologic factors of disease by appropriate oral hygiene. In accordance, we demonstrate that the frequencies of periodontopathogens were strikingly lower in the control group, as previously described (3).

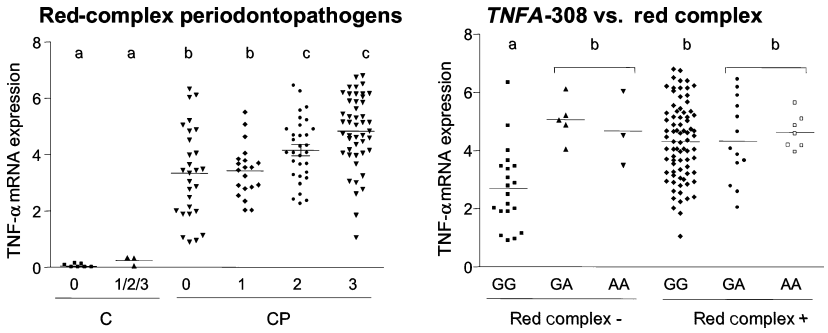

Fig. 4. Quantitative assessment of tumor necrosis factor-alpha mRNA expression in the presence or absence of red-complex periodonthogens associated with the genotypes in the chronic periodontitis group. Control subjects and chronic periodontitis patients were subjected to periodontal examination, and the *TNFA* −308 single nucleotide polymorphism genotype was determined using restriction fragment length polymorphism analysis. Total RNA was extracted from gingival tissues, and the levels of tumor necrosis factor-alpha mRNA were measured quantitatively using real-time polymerase chain reaction. The results are presented as expression of the individual mRNAs, with normalization to beta-actin, using the cycle threshold method. The presence of the red-complex periodontopathogens (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*) was investigated by real-time polymerase chain reaction. The graphs depict the expression of tumor necrosis factor-alpha in chronic periodontitis patients regarding the presence of 0, 1, 2 or 3 species of red-complex periodontopathogens and also regarding their *TNFA* −308 single nucleotide polymorphism genotype concomitantly with detection of red-complex periodontopathogens; $p < 0.05$ (analysis of variance): different letters indicate statistical significance. C, control; CP, chronic periodontitis; TNF-α, tumor necrosis factor-alpha.

Notwithstanding the controversies regarding genetic association studies, evaluation of the presumed functionality of the *TNFA* −308 single nucleotide polymorphism in a periodontal environment could be helpful in understanding its putative role in this pathology. Our results following analysis of the influence of the *TNFA* −308 single nucleotide polymorphism on tumor necrosis factor-alpha levels demonstrate a slight increase in mean tumor necrosis factor-alpha expression in GA and AA groups when compared with patients who have the GG genotype. In accordance, the occurrence of the A allele in the *TNFA* −308 single nucleotide polymorphism was associated with an increased level of tumor necrosis factor-alpha *in vitro* in response to different stimuli (9), and also in pathological conditions such as rheumatoid arthritis (8,11,35–37), which share with periodontitis characteristics such as the chronic nature of the inflammatory reaction associated with bone-resorptive activity (38). Interestingly, the *TNFA* −308 polymorphism is also associated with the severity of rheumatoid arthritis (37), which recently has been found to be clinically associated with periodontal diseases (38,39), suggesting that these pathologies could share genetic susceptibility/resistance patterns.

It is also important to consider that the presence of specific periodontopathogens could also modulate tumor necrosis factor-alpha levels. In fact, *P. gingivalis*-, *T. forsythia*- and *T. denticola*-positive sites presented a significantly higher expression of tumor necrosis factor-alpha when compared with the negative sites, and the loads were positively correlated with tumor necrosis factor-alpha mRNA levels. In accordance, *P. gingivalis* can induce tumor necrosis factor-alpha production by diverse cell types (15,16,40) and was also associated with elevated tumor necrosis factor-alpha levels in periodontal lesions (41,42). Indeed, tumor necrosis factor-alpha mediates *P. gingivalis*-induced bone resorption in mice (43). Similarly, *T. forsythia* and *T. denticola* have been associated with tumor necrosis factor-alpha production *in vitro* and *in vivo* (15,42). *P. gingivalis*, *T. forsythia* and *T. denticola* were associated with higher scores of disease-severity parameters, in accordance with previous studies (18,26,27). Interestingly, the evaluation of the presence of individual red-complex periodontopathogens resulted in similar results, probably because of their simultaneous occurrence in the majority of the patients. Indeed, the red-complex periodontopathogens were shown to exist as a consortium (44), and to exhibit synergistic virulence in a rat model of polymicrobial periodontal infection (45). In accordance, our results demonstrate a gradual increase in tumor necrosis factor-alpha levels associated with the simultaneous occurrence of red-complex species, suggesting a synergistic pathogenic pro-inflammatory role for these bacteria. On the other hand, *A. actinomycetemcomitans*-positive sites showed a slight decrease in tumor necrosis factor-alpha expression, but such a difference did not reach statistical significance. While some studies describe that *A. actinomycetemcomitans* can induce tumor necrosis factor-alpha production *in vitro* and *in vivo* (16,46), others describe that *A. actinomycetemcomitans* has immunosuppressive properties that could inhibit the production of the inflammatory cytokine tumor necrosis factor-alpha (47–49). However, the low frequency of *A. actinomycetemcomitans* in our sample does not permit definitive conclusions to be reached, and further studies are required to evaluate its putative role in the modulation of tumor necrosis factor-alpha levels.

Taken together, our data suggest that both *TNFA* −308 and the red-complex bacteria are associated with higher tumor necrosis factor-alpha levels. However, in the view of possible overlapping effects (i.e. a red-complex-positive A-allele carrier), we next evaluated their individual roles in the modulation of tumor necrosis factor-alpha expression. Our results demonstrate that in the absence of red-complex bacteria (individually and as a group), GA/AA carriers presented significantly higher levels of tumor necrosis factor-alpha than patients with the GG genotype. When red-complex bacteria (individually and as a group) positive subjects were analyzed, GG and GA/AA groups pre-

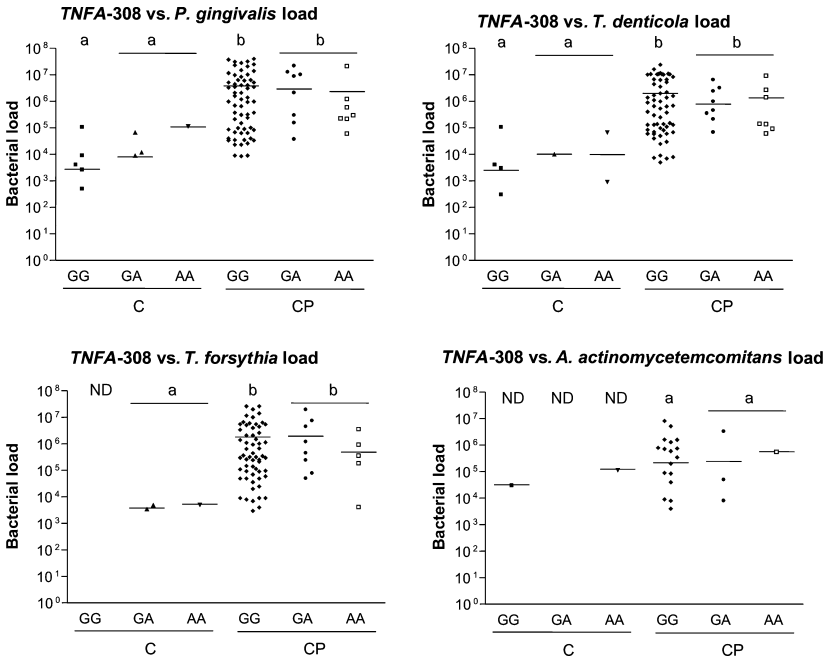

Fig. 5. Quantitative assessment of the periodontopathogen load in the different tumor necrosis factor-alpha (*TNFA*) −308 genotype groups. Control subjects and chronic periodontitis patients were subjected to periodontal examination, and the *TNFA* −308 single nucleotide polymorphism genotype was determined using restriction fragment length polymorphism analysis. The load of the periodontopathogens *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans* was determined by real-time polymerase chain reaction. The graphs depict the bacterial load in control subjects and chronic periodontitis patients regarding their *TNFA* −308 single nucleotide polymorphism genotype. *ND, not determined (subgroup $n = 0$ or $n = 1$); $p < 0.05$ (analysis of variance): different letters indicate statistical significance. C, control; CP, chronic periodontitis; TNF-α, tumor necrosis factor-alpha.

sented similar levels of tumor necrosis factor-alpha, equivalent to those seen in GA/AA carriers in the absence of the red-complex periodontopathogens. Therefore, our data demonstrate that both red-complex periodontopathogens and the *TNFA* −308 single nucleotide polymorphism can independently lead to a high expression of tumor necrosis factor-alpha in diseased periodontium. Interestingly, no additive effect was found when both factors were concurrent, suggesting that both factors can independently reach an individual plateau of maximum tumor necrosis factor-alpha expression. The positive association of both *TNFA* −308 single nucleotide polymorphism and red-complex periodontopathogens with tumor necrosis factor-alpha levels in diseased periodontium demonstrated herein is reinforced by the fact that smokers were specifically excluded from our sample, as smoke characteristically interferes in the host response (which includes the modulation of tumor necrosis factor-alpha levels) (50,51). However, it is also important to consider that other periodontopathogens, and even other pathogens recently associated with periodontal disease, such as viruses, may also influence the levels of tumor necrosis factor-alpha in diseased periodontium (52).

Interestingly, *TNFA* −308 genotypes were also not associated with a different frequency or load of periodontopathogens. Similarly, TNFA genotypes were not associated with the load or with increased odds of detecting *A. actinomycetemcomitans*, *P. gingivalis* or *T. forsythensis* in periodontal pockets (53–55). While the differential tumor necrosis factor-alpha production by mice strains selected for minimal or maximal inflammatory reactions [acute inflammatory reaction (AIR)min and AIRmax strains] is not associated with the periodontal bacterial load (56), the absolute deficiency of tumor necrosis factor-alpha signaling impairs the control of experimental periodontal infection in mice (5). Therefore, our data suggest that hyperinflammatory single nucleotide polymorphisms did not confer an advantage in the host response against periodontopathogens, and further studies are required to investigate the existence of a putative optimum level of host response, which could restrain the infection with a minimum damage to the host tissues.

The possible role of single nucleotide polymorphisms on periodontitis outcome remains controversial, mainly as a result of divergent criteria for the selection of disease and control groups, small size samples and the lack of observance of covariates such as smoking and specific bacteria (7,34). In a study designed to minimize some of these limitations, our data demonstrate that both *TNFA* −308 genotypes and red-complex periodontopathogens can modulate the levels of tumor necrosis factor-alpha in diseased tissues of nonsmoking chronic periodontitis patients and, consequently, are potentially involved in the determination of disease outcome. Accordingly, meta-analysis studies demonstrate that the observance of covariates is important to unravel the possible association of *TNFA* −308 with different pathological conditions (10–12). Interestingly, *TNFA* −308 single nucleotide polymorphism *in vitro* can increase tumor necrosis factor-alpha production up to fivefold (8), while a lower increase rate was demonstrated *in vivo* (57–59), in accordance with our results. Indeed, periodontitis is a complex trait disease, in which several other immunoregulatory cytokines and mechanisms are involved (1,7), and even in experimental periodontitis in knockout mice, in which a given cytokine is completely absent, the disease severity is usually found to be partially, but not completely, increased/decreased (5,22,60). Therefore, it is reasonable to expect that single nucleotide polymorphisms, even those functional as *TNFA* −308, exert a significant, but not a major role in dis-

ease progression, as described for other pathologies (57–59). The first steps in solving the puzzle regarding the contribution of genetic make-up and microbes to the immunopathogenesis of periodontal disease have been achieved, but further studies are required to understand their exact roles in periodontitis outcome.

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