

Genetic markers of tumour necrosis factor α in aggressive and chronic periodontitis

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Abstract

Aim: Tumour necrosis factor α (TNF α) plays an important role in the pathogenesis of periodontitis. TNF α production is influenced by gene polymorphisms. The aim of this study was to evaluate links between genetic variants and chronic/aggressive periodontitis in a multivariate model.

Subjects: One hundred and twenty-three periodontitis patients (chronic: $n = 54$, aggressive: $n = 69$) and 52 healthy controls without periodontitis were included in the study.

Material and Methods: Single nucleotide polymorphisms (SNPs) c. –308G > A, c. –238G > A and haplotypes were analysed by a polymerase chain reaction with sequence-specific primers (PCR-SSP). The clinical investigation included smoking status, plaque and bleeding indexes, pocket depth and attachment loss.

Results: *Prevotella intermedia* occurred more frequently in individuals positive for the –308GG/–238GG haplotype combination (Odds Ratio = 2, 95% Confidence interval: 1.1–3.7, $p = 0.037$, $1 - \beta = 61\%$). In binary logistic regression analyses, this TNF α haplotype could not be shown to be associated with periodontitis considering smoking, age, gender and approximal plaque index or subgingival bacterial colonization as confounding factors.

Conclusions: Although the genetic background of TNF α could be shown to be associated with subgingival colonization with *P. intermedia*, there is no evidence that it is an independent risk factor for periodontitis in multivariate models.

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Periodontitis is a chronic inflammatory disorder of the periodontal supportive tissue of teeth. The genetic impact on the aetiology of periodontitis was

proven in previous clinical studies (Michalowicz et al. 2000). Although the occurrence of periodontal bacteria is considered to be the main cause of periodontitis, the disease may also be affected by certain characteristics of the individual immune system (Honda et al. 2006).

TNF α is a potent pro-inflammatory cytokine and the individual cytokine production is discussed as an important factor involved in the aetiology of periodontal disease and could have an influence on the therapeutical outcome (D'Aiuto et al. 2005, 2007, Feng et al. 2006). TNF α is released at the sites of inflammation. It influences the immune response via the activation of cells, such

as endothelial cells and gingival fibroblasts, the induction of cytokine production, the up-regulation of adhesion molecules, as well as the stimulation of matrix metalloproteinases (Borish & Steinke 2003, Graves & Cochran 2003, Hosokawa et al. 2005). In animal models, it could be shown that due to the up-regulation of metalloproteinase-1, TNF α potently promotes alveolar bone resorption, which is a crucial process in periodontitis (Hong et al. 2004, Lima et al. 2004).

Additionally, in a clinical study, Have-mose-Poulsen et al. (2005) revealed that patients with aggressive periodontitis (AP) had an increased plasma concentration of TNF α . Furthermore, patients

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with chronic or AP had higher levels of TNF α in their gingival crevicular fluid, causing more severe inflammation in periodontitis lesions (Kurtis et al. 2005). Moreover, the greater the attachment loss, the more the bleeding on probing (BOP), and the higher the plaque index in patients with chronic periodontitis (CP) and type 2 diabetes, the higher the TNF α concentration (Engebretson et al. 2007). These findings show a correlation between disease severity and TNF α expression as an indicator of a weakened immune response (Ikezawa et al. 2005, Engebretson et al. 2007).

It is generally accepted that the individual ability to regulate TNF α is of paramount importance for a protective or a destructive immune response (Rahman & McFadden 2006). This study showed that TNF α expression may be regulated by genetic variants located mainly in the promoter of this gene. Many different single nucleotide polymorphisms (SNPs) have hitherto been identified in the promoter region of TNF α (Deshpande et al. 2005). In several clinical studies, the importance of the genetic background of the patient for TNF α regulation was emphasized in various inflammatory diseases including rheumatoid arthritis, cardiovascular disease and Crohn's disease as well as in cell models (Cuenca et al. 2003, Gonzalez et al. 2003, Schulz et al. 2004, 2006; for a review, see Loos et al. 2005). The influence of polymorphic sites on its regulation in the TNF α promoter region was also studied

in periodontitis patients. In a clinical study, an association between c. -308G > A polymorphism and TNF α production was proven in adult patients with advanced periodontitis (Galbraith et al. 1998). However, this association could not be confirmed in patients with AP (Perez et al. 2004). Moreover, there was no evidence of a dependence of the c. -238G > A polymorphism on TNF α expression in adults with periodontitis (Galbraith et al. 1998).

Bearing in mind a possible link between genetic variants and expression, the polymorphisms in TNF α were considered to be risk factors in periodontitis. Several clinical studies were carried out to address this issue. However, the results obtained from these studies are controversial (Kornman et al. 1997, Galbraith et al. 1999, Endo et al. 2001, Shapira et al. 2001, 2005, Craandijk et al. 2002, Lin et al. 2003, Soga et al. 2003, Folwaczny et al. 2004, Donati et al. 2005, Sakellari et al. 2006, Scapoli et al. 2007).

Little is known about the complex interaction of the genetic background of TNF α using analyses of both genetic markers (genotype, haplotype and haplotype combination) and clinical markers of periodontitis. Therefore, the initial aim of this study was to investigate the impact of TNF α polymorphisms c. -308G > A and c. -238G > A, including their haplotypes, on the occurrence of generalized CP and generalized AP. The second aim was to evaluate the influence of genetic variants of TNF α considering established confounders for periodontitis, such as age, gender,

smoking, plaque index and the occurrence of periodontal bacteria, in a multivariate model.

Material and Methods

Study population

One hundred and seventy-five unrelated persons of the same Caucasian origin from Central Germany were involved in our study consecutively. The patient groups ($n = 123$) comprised 54 patients with generalized CP and 69 patients with generalized AP. The control group included 52 periodontitis-free healthy participants (demographic data are given in Table 1).

In general, we excluded persons who were pregnant, had a drug-induced gingival hyperplasia or received antibiotics in the last 6 months. Moreover, persons with chronic usage of anti-inflammatory drugs were excluded. Furthermore, subjects with a history of HIV infection or with acute infection of the oral cavity (e.g. herpetic gingivostomatitis) were excluded. Other criteria for exclusion were oral pemphigus or pemphigoid, type I or type II diabetes mellitus, coronary heart disease, rheumatic diseases, lupus erythematosus, Behcet disease and Crohn's disease.

Patients with periodontitis and individuals without periodontitis were recruited at the Department of Operative Dentistry and Periodontology irrespective of concomitant risk factors. Two independent periodontists (S. R. and J. K.) made the clinical diagnosis.

Table 1. Clinical characteristics of the groups of patients and healthy controls

Variable	Chronic periodontitis ($n = 54$)	Aggressive periodontitis ($n = 69$)	No periodontitis ($n = 52$)
<i>Demographic parameter</i>			
Average age (years)	48.5 \pm 9.8	41 \pm 1	44.6 \pm 11
Female (%)	68.5	63.8	55.8
Current smoker (%)	25.2	33.3	25
Past smokers (%)	9.3	8.7	5.8
<i>Clinical parameter</i>			
Approximal plaque index (%)	59.1 \pm 26.3*	53.8 \pm 29.4*	43.1 \pm 8.7
Bleeding on probing (%)	67.7 \pm 25.1*	78.1 \pm 23.7*	45.4 \pm 24
Clinical probing depth (mm)	5.1 \pm 1.1*	6.4 \pm 5.7*	2.7 \pm 0.9
Clinical attachment loss (mm)	5.8 \pm 1.4*	6.6 \pm 1.6*	3 \pm 1
<i>Individual occurrence of periodontal bacteria in subgingival pockets</i>			
<i>Actinobacillus actinomycetemcomitans</i> (%)	25.9	42**	15.4
<i>Porphyromonas gingivalis</i> (%)	87**	79.7**	15.4
<i>Prevotella intermedia</i> (%)	61.1**	65.2**	25
<i>Tannerella forsythensis</i> (%)	96.3**	87**	59.6
<i>Treponema denticola</i> (%)	98.1**	87**	63.5

* $p < 0.05$ in comparison with the control group (normal distribution of all metric values \rightarrow statistical evaluation with parametric tests).

** $p < 0.05$ in comparison with the control group (Chi2-test or Fisher's exact test if $n < 5$).

During the course of the anamnesis, the patients and controls were asked about any current or past diseases and whether they were taking medication or smoked. A person who smoked a minimum of one cigarette per day was considered to be a smoker. The clinical assessment included determination of the approximal plaque index (API) (Lange et al. 1977) and assessment of BOP. The measurements for both maximal clinical probing depth (PD = distance between the gingival margin and the bottom of the pocket) and maximum clinical attachment loss (CAL = distance between the cemento-enamel junction and the bottom of the pocket) were taken at six sites around each tooth. Maximum values were considered. Inclusion criteria for patients suffering from generalized CP were as follows: $\geq 30\%$ of the teeth were affected, PD ≥ 4 mm and amount of CAL was consistent with the presence of mineralized plaque. Generalized AP was characterized as follows: patients were generally healthy, age of onset of disease < 35 years, attachment loss of 4 mm or more in at least 30% of the teeth, at least three of the affected teeth were not first molars and incisors and the severity of attachment loss was inconsistent with the amount of mineralized plaque. The group of periodontally healthy individuals fulfilled the following criteria: age ≥ 30 years, PD ≤ 3.5 mm and no gingival recession due to periodontitis. A CAL > 3.5 mm as a consequence of traumatic tooth brushing, overhanging dental fillings, orthodontic therapy, etc. was not considered to be a case of periodontitis.

Clinical details of the patient groups are demonstrated in Table 1

All participants gave their written consent to participation in this study. The study was approved by the ethics committee of the Medical School of the Martin-Luther University of Halle-Wittenberg. The investigations were carried out in accordance with the ethical guidelines of the "Declaration of Helsinki" and its amendment in "Tokyo and Venice".

Molecular biological assessment of periodontal bacteria in the subgingival pockets

Microbial samples were taken before subgingival scaling was carried out.

They were collected from the deepest pocket of each quadrant by inserting a sterile paper point for 20 s. Bacterial plaque samples, taken from each patient, were pooled in one tube. Preparation of bacterial DNA was carried out using the QIAamp[®] DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's manual. For specific amplification of *Actinobacillus actinomycescomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis* and *Treponema denticola* DNA, the micro-Ident[®] test of HAIN-Diagnostik (Nehren, Germany) based on an alkaline phosphatase-mediated staining reaction was used. PCR was performed in a personal cycler (Biometra, Göttingen, Germany) according to the following program: 5 min. 95°C; 10 cycles: 30 s 95°C, 2 min. 58°C; 40 s 70°C, 20 cycles: 25 s 95°C, 40 s 53°C, 40 s 70°C; and 8 min. 70°C.

The PCR products were hybridized to a strip (hybridized with DNA sequences of each bacteria as well as a positive control) and the occurrence of bacteria was visually evaluated by means of coloured bands. Two positive controls for the amplification reaction and for hybridization were included in the test.

Genetic studies on TNF α -polymorphisms

For genetic investigations, fresh venous EDTA-blood was obtained from the test persons. The preparation of genomic DNA was carried out using a QIAamp[®] blood extraction kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's manual.

The detection of genotypes and haplotypes of TNF α SNPs (c. -308G>A and c. -238G>A) was carried out using the CYTOKINE Genotyping array CTS-PCR-SSP Tray kit (Collaborative Transplant Study, Department of Transplantation Immunology of the University Clinic of Heidelberg, Germany). A fragment of 440 bp of the human C-reactive protein was co-amplified as a positive control for each PCR. The PCRs were performed with sequence-specific primers for the detection of possible haplotypes and pipetted and lyophilized in thin-walled plastic 96-well PCR trays. For every PCR, 10 μ l of a Mastermix containing 1 U Taq-Polymerase (Invitex, Berlin, Germany), 100 ng genomic DNA, 5% glycerol and PCR reaction buffer were added. PCR was performed in an

Eppendorf Mastercycler Gradient (Eppendorf, Wesseling-Berzdorf, Germany) (2 min. 94°C; 10 cycles: 15 s 94°C, 1 min. 64°C, 30 s 72°C; 20 cycles: 15 s 94°C, 50 s 61°C, 30 s 72°C). After agarose gel electrophoresis, the resulting pattern was evaluated visually.

All sequence data were in accordance with the sequence of the human TNF α gene (Genbank: accession number Z15026). According to db SNP, the identification numbers of the SNPs were rs1800629 (c. -308G>A) and rs361525 (c. -238G>A), respectively.

Statistical evaluation

Statistical analyses were carried out using the program SPSS 15.0. *p*-Values of ≤ 0.05 were considered to be significant. The genotype distributions of the polymorphisms were tested in accordance with the Hardy-Weinberg equilibrium. Categorical variables were plotted in contingency tables and evaluated using Chi square analysis and Yates continuity correction. If $n < 5$ Fisher's exact test was performed. Metric parameters are presented as mean \pm standard error (SE). These data were analysed using the Kolmogorov-Smirnov test (test of normal distribution). For the statistical evaluation, the Student's *t* test or one-way ANOVA (normally distributed values) and the Mann-Whitney *U*-test or the Kruskal-Wallis test (values not distributed normally) were used. For power evaluation, the program nQuery Advisor 4.0 was applied. Binary logistic regression analysis was used for investigating the impact of polymorphic variants in the TNF- α gene on the development of chronic and/or AP considering known covariates for periodontitis such as age, gender, smoking, API and subgingival microbiota. Possible interactions between variables included in this complex model were considered.

Results

Clinical assessment

All individuals involved in the study were evaluated according to their age, gender, and smoking status. On comparing the patient groups with the periodontitis-free healthy controls, no statistically significant differences in age, gender and smoking status could be detected.

The periodontal conditions such as API, BOP, PD, and CAL were significantly more pronounced in the two

Table 2. Influence of genotype, allele and haplotype distribution of tumour necrosis factor α (TNF α) SNPs c. – 308G>A and c. – 238G>A in dependence on the occurrence of chronic and aggressive periodontitis

	All patients (n = 123)	CP (n = 54)	AP (n = 69)	No periodontitis (n = 52)	p	Power 1 – β
Genotypes						
c. – 308G>A						
GG (%)	66.7	68.5	65.2	65.4		
AG (%)	28.5	29.6	27.5	34.6		
AA (%)	4.9	1.9	7.2	0	0.302	44%
c. – 238G>A						
GG (%)	91.9	90.7	92.8	84.6		
AG (%)	7.3	7.4	7.2	15.4*		
AA (%)	0.8	1.9	0	0	0.267	40%
Alleles						
c. – 308G>A						
G (%)	80.9	83.3	79	82.7		
A (%)	19.1	16.7	21	17.3	0.633	12%
c. – 238G>A						
G (%)	95.5	94.4	96.4	92.3		
A (%)	4.5	5.6	3.6	7.7	0.384	22%
Haplotype combinations						
c. – 308G>A/c. – 238G>A						
GG–GG (%)	58.5	59.3	58	50		
GG–AG (%)	29.3	31.5	27.5	34.6		
GG–GA (%)	6.5	5.6	7.2	15.4*		
AG–AG (%)	4.9	1.9	7.2	0		
GA–GA (%)	0.8	1.9	0	0	0.203	64%
Haplotypes carrying mutant alleles (%)	41.5	40.7	42	50	0.577	14%
Haplotypes						
GG (%)	76.4	77.8	75.4	75		
AG (%)	19.5	17.6	21	17.3		
GA (%)	4.1	4.6	3.6	7.7*	0.643	21%

*An almost two-fold increase in the prevalence of genotype and haplotype carriers among healthy subjects compared with patients suffering from periodontitis. CP, chronic periodontitis; AP, aggressive periodontitis.

patient groups (Table 1). In both groups of patients, there was an increase in the amount of bacteria. However, there was no significant difference with respect to *A. actinomycetemcomitans* between patients with CP and the control group.

Genetic investigation on TNF α SNPs c. – 308G>A and c. – 238G>A

Bivariate analyses

The importance of the genetic variants of TNF α , c. – 308G>A and c. – 238G>A considering its genotype and haplotype distribution in periodontitis was assessed. The genotype distributions in the group of periodontal healthy individuals (SNPs c. – 308G>A and c. – 238G>A) were in keeping with the Hardy–Weinberg equilibrium. There was no evidence of a link between the genotype or haplotype combination frequencies on comparing the patient group (AP+CP) with periodontal healthy individuals. After subdividing the patients according to

their clinical status (AP and CP groups), no significant association could be found either (Table 2). The statistical power (1 – β) of the corresponding evaluations ranged from 12% to 64% (Table 2).

Possible links between genetic variants of TNF α and microbiological findings (occurrence of periodontal bacteria in subgingival pockets, including *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythensis* and *T. denticola*) and clinical data (API, BOP, PD and CAL) were assessed.

The occurrence of *P. intermedia* was associated with c. – 238G>A genotype ($p = 0.032$) and c. – 308G>A/c. – 238G>A haplotype combination ($p = 0.025$). Only the connection between *P. intermedia* and c. – 308G>A/c. – 238G>A haplotype combination remained statistically significant after Yates correction ($p = 0.037$). Carriers of the wild-type haplotype combination (GG–GG) showed a significantly higher prevalence of *P. intermedia*. (Table 3). The power (1 – β) of this evaluation was 61%. No other significant links

Table 3. Association of the individual genotype/haplotype distribution and the occurrence of five subgingival periodontal bacteria in the total study group. Only for *Prevotella intermedia* could a significant association be proven

	<i>P. intermedia</i> (%)
c. – 238G>A	
GG (n = 156)	54.8
AG+AA (n = 18)	27.8
p	0.058*
Odds ratio (95% CI)	3.1 (1.1–9.2)
Power (1 – β)	57%
c. – 308G>A	
GG (n = 116)	54.3
AG+AA (n = 58)	46.6
p	0.421*
Odds ratio (95% CI)	1.36 (0.72–2.6)
Power (1 – β)	15%
Haplotype combinations c. – 308G>A/c. – 238G>A	
GG–GG (n = 98)	59.2
Haplotype combinations carrying at least one mutant allele (n = 76)	42.1
p	0.037*
Odds ratio (95% CI)	2 (1.1–3.7)
Power (1 – β)	61%

*Yates correction.

between TNF α polymorphisms and subgingival bacterial colonization could be proven in the entire study group.

However, a link between the clinical outcome of periodontitis, namely BOP, and the TNF α haplotype could be detected in the group of patients suffering from CP. Subjects carrying mutant haplotypes in comparison with carriers of wild-type haplotypes had an increased mean percentage of BOP (Student's *t*-test: $p = 0.022$, GG–GG (BOP %): 61.4 ± 26.8 , haplotypes carrying mutant alleles (BOP %): 77.4 ± 18.9). Other markers of periodontitis investigated, including API, CAL and PD, could not be proven to be associated with the TNF α haplotype.

In addition, no dependence of other factors, known to affect periodontitis, on the genetic background of TNF α (including age, gender and smoking status) could be determined.

Multivariate analyses (binary logistic regression analysis)

Possible interactions of the genetic background and confounding factors on the occurrence of periodontitis were investigated by means of logistic regression analysis. Because there is a correlation between API and subgingival

Table 4. Forward stepwise binary logistic regression analyses investigating the impact of the genetic background of tumour necrosis factor α (TNF α) (haplotype combinations GG–GG versus GG–AG+GG–GA+AG–AG) on periodontitis considering the occurrence of periodontal bacteria (a) and age, gender, smoking and approximal plaque index (b)

Significant variables	Regression coefficient	SE	<i>p</i> -value	Odds ratio	95% CI	
					lower	upper
(a) Patients with periodontitis versus healthy subjects						
<i>Porphyromonas gingivalis</i>	3.05	0.46	<0.001	21	8.5	52
<i>Prevotella intermedia</i>	1.04	0.46	0.021	2.84	1.2	6.9
Haplotype combination GG–GG			0.933			
Patients with chronic periodontitis versus healthy subjects						
<i>Prevotella gingivalis</i>	3.6	0.56	<0.001	36.1	12.1	108.1
Haplotype combination GG–GG			0.705			
Patients with aggressive periodontitis versus healthy subjects						
<i>Porphyromonas gingivalis</i>	2.8	0.5	<0.001	16.5	6.2	44
<i>Actinobacillus actinomycetemcomitans</i>	1.1	0.5	0.023	3.1	1.2	8.2
Haplotype GG–GG			0.884			
(b) Patients with periodontitis versus healthy subjects						
API	0.021	0.007	0.004	1.02	1.01	1.04
Haplotype combination GG–GG			0.191			
Patients with chronic periodontitis versus healthy subjects						
API	0.031	0.01	0.002	1.03	1.01	1.05
Haplotype combination GG–GG			0.127			
Patients with aggressive periodontitis versus healthy subjects						
API	0.017	0.008	0.031	1.02	1.02	1.03
Haplotype GG–GG			0.430			

API, approximal plaque index.

bacterial colonization (*P.g.*: $r = 0.31$, $p < 0.001$; *P.i.*: $r = 0.195$, $p = 0.013$; *T.f.*: $r = 0.241$, $p = 0.002$; *T.d.*: $r = 0.249$, $p = 0.001$), two separate statistical analyses were performed. In the initial step, the effect of periodontal bacteria and genetic background characterized by the haplotype combination (GG–GG versus GG–AG+GG–GA+AG–AG) was evaluated (Table 4a). There was no proof of an independent influence of the haplotype constellation on the prevalence of periodontitis (periodontitis in general, chronic and AP). The occurrence of periodontal bacteria was the strongest predictor for periodontitis in this model.

In a second complex model considering further possible confounding factors including age, gender, smoking status and API as demographic clinical features, the influence of the genetic background of TNF α was tested. Once again, the TNF α haplotype combination did not account for the development of CP, AP, or periodontitis in general (Table 4b).

Discussion

Recent research has established that inflammation plays an important role in the development of a variety of complex diseases including periodontitis. The proinflammatory cytokine TNF α is involved in different inflammatory pro-

cesses modulating the development of periodontitis, including regulation of bone resorption and fibroblast cell proliferation (Loos et al. 2005). Furthermore, the TNF α level is increased in the gingival crevicular fluid of patients with periodontitis as well as in inflamed periodontal tissues (Loos et al. 2005). Therefore, the gene that encodes for TNF α including its genetic characteristics is considered to be a candidate gene for periodontitis. In this study, the influence of the genetic background of the pro-inflammatory cytokine TNF α on the occurrence of periodontitis and its clinical features was investigated. For the first time, the importance of the combination of TNF α haplotypes (c. –308G>A & c. –238G>A) was assessed in a complex model in which confounders of periodontitis were considered.

Clinical assessment

Our study group comprised clinically well-characterized periodontitis patients with generalized CP or generalized AP. In the control group, the individuals were from the same geographical region and were not suffering from periodontitis. Because periodontitis increases with age and usually occurs after the age of 30, only controls aged 30 years and over were included in the

study (Albandar 2005, Timmerman & van der Weijden 2006).

Other clinical features of periodontitis were taken into consideration, including API, BOP, clinical PD, and CAL. As expected, both patient groups exhibit the more severe clinical characteristics. Analyses of the higher prevalence of periodontal bacteria in subgingival pockets in patients with chronic or AP confirmed previously established results (Pihlstrom et al. 2005). Moreover, the higher frequency of *A. actinomycetemcomitans* among patients with AP compared with patients suffering from CP was in accordance with the results obtained previously (Cortelli et al. 2005). The most interesting finding is the occurrence of periodontal bacteria among healthy controls. Although the subgingival bacterial colonization is less pronounced in these controls, it is worth noting that these subjects have no clinical signs of periodontitis despite bacterial colonization. This result indicates the importance of other factors affecting disease, for example genetic markers.

Genetic Investigations

The investigations presented here are designed as a case–control association study in order to investigate the association of the genetic variants of TNF α and

the occurrence of periodontitis including its clinical features. As reviewed by Cordell and Clayton (2005) and Wang et al. (2005), it has been realized that genetic susceptibility to complex disorders involves many genes, most of which exhibit only small effects. Furthermore, the underlying genetic effect can only be assessed in association studies if the polymorphism has a causal role, the variant is in close vicinity to a causal polymorphism or the association is only caused by a stratification of the population. Therefore, association studies are the most powerful tools when investigating functional important genetic variants of proven susceptibility genes in a population of highly selected unrelated individuals. This requires high attention in selecting the control probands as well as considering confounding factors. Several case-control studies are conducted to assess the importance of the functionally important TNF α variants also investigated in the present study (Yoshie et al. 2007). However, studies on this subject are controversial. Whereas a few studies could prove that TNF α polymorphisms are factors affecting disease (Galbraith et al. 1999, Soga et al. 2003, Lin et al. 2003; for a review, see Shapira et al. 2005, Loos et al. 2005), many researchers could not confirm a link between genetic variants and periodontitis (Endo et al. 2001, Shapira et al. 2001, Craandijk et al. 2002, Fassmann et al. 2003, Folwaczny et al. 2004, Donati et al. 2005; for a review, see Loos et al. 2005). The controversial results from different studies could partly be explained by the different clinical and statistical settings as well as by ethnic differences in genotype distributions. Ethnic variances in TNF α genotype distribution were reported by others (Cuenca et al. 2001). In contrast to other studies (Craandijk et al. 2002, D' Aiuto et al. 2004), Caucasians from an accurately defined geographical region of Central Germany were included in this case-control study in order to diminish the loss of power due to ethnic variability. Furthermore, considerable attention was paid to the clinical selection criteria of patients and controls. Only patients with severe periodontitis were evaluated in the present study. Because moderate and mild forms of periodontitis are most probably influenced by a greater variety of exogenous and endogenous factors, the clinical selection presented here is more reasonable for the evaluation of

genetic factors. In this study, we could not detect a statistically significant influence of the genetic variants of TNF α , c. -308G>A and c. -238G>A including the corresponding haplotypes on the occurrence of aggressive or CP in bivariate analyses (Table 2). However, as pointed out in Tables 2 and 3, the power of the statistical evaluation is comparatively low. Depending on the distribution of the markers investigated in the present study, the power ranged from 12% to 64%. This implies a definite level of uncertainty of the results presented.

A statistical power of approximately 80% is considered to be required in order to obtain statistically reliable data. For investigation of the TNF α genotype and haplotype distribution in periodontitis, this would imply the analysis of approximately 350 patients and controls (according to statistical evaluation using nQuery Advisor 4.0). However, to our knowledge, up to now, no clinical association study addressing the impact of genetic variants on the occurrence of periodontitis has such a large study size (Galbraith et al. 1999, Endo et al. 2001, Craandijk et al. 2002, Fassmann et al. 2003, Soga et al. 2003, Folwaczny et al. 2004, Donati et al. 2005). In order to obtain scientifically more substantiated results, our preliminary results should be indicative for prospective studies investigating larger cohorts. Because the data presented are collected from highly selected patients and controls of a distinct geographical region, the results can be considered to be valid for Caucasians of Central Germany. However, the opportunity of extrapolation to the general population is not rational.

By investigating possible links between the genetic constellation of TNF α and risk factors and symptoms of periodontitis, it could be shown that CP patients carrying the wild-type haplotype combination GG-GG had a decreased mean percentage of BOP. At the same time, this haplotype increased the OR for the subgingival colonization with *P. intermedia* (Table 3). Thus, the TNF α haplotype GG-GG might well indicate a decreased inflammatory response, implying a decrease in the host's bacterial defence mechanism.

The possibility of the higher prevalence of this periodontal pathogen for wild-type carriers having a functional basis is yet to be clarified. In previous studies, possible correlations of TNF α

expression and the occurrence of periodontal bacteria were investigated. It was shown that periodontal bacteria may induce the expression of TNF α in vivo or in vitro blood models (Bodet et al. 2006a, b). Otherwise, nothing is known about the impact of genetic variants on the occurrence of periodontal bacteria. Only the study by Trevilatto et al. (2002) evaluated the genetic variant c. -308G>A in relation to the occurrence of periodontal bacteria in a Brazilian family with AP. Because this family only comprised wild-type carriers, it was not possible to detect a link between this genetic variant and subgingival bacteria.

If periodontitis is affected by a variety of other factors including gender (Reichert et al. 2003, Timmerman & van der Weijden 2006), smoking (Palmer et al. 2005, Pihlstrom et al. 2005, Heasman et al. 2006) or age (Albandar 2005, Timmerman & van der Weijden 2006), a complex model should be used for better evaluation of the genetic impact. Therefore, for the first time, we investigated the influence of the TNF α haplotype combination (c. -308G>A and c. -238G>A) considering subgingival bacterial colonization as well as age, gender, smoking, and API as confounding factors in binary logistic regression analysis. However, the TNF α haplotype combination could not be proven to be an independent risk factor in periodontitis in general and chronic or AP. Accordingly, no influence of TNF α promoter polymorphisms (c. -1031T>C, c. -863C>A, c. -857C>T, c. -308G>A) on the occurrence of periodontitis was detected in several studies (Yoshie et al. 2007). In our multivariate models, subgingival bacterial colonization and API were shown to be risk factors in periodontitis.

Conclusion

Despite the limitations of clinical association studies, our results support the conclusion that the TNF α -308GG/-238GG haplotype combination GG-GG has to be considered to be a modulating factor for clinical features of periodontitis including subgingival colonization with *P. intermedia* and BOP. However, in a multivariate model in which confounders for periodontitis were taken into consideration, the genetic background of TNF α could not be proven

to be an independent risk factor for periodontitis in Caucasians from Central Germany.

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Clinical Relevance*Scientific rationale for the study:*

There has been an increasing demand for a stable prognostic and therapeutically relevant genetic marker for a better assessment of an adequate periodontal therapy.

Polymorphisms in the pro-inflammatory cytokine were shown to affect TNF α -production. Therefore, TNF α polymorphisms might be indicative of both generalized aggressive and severe CP.

Main findings: The genetic background of TNF α (haplotypes of

c. – 308G > A, c. – 238G > A) was not associated with the occurrence of periodontitis in a complex risk model.

Practical implications: TNF α haplotype cannot be considered to be a risk marker for periodontitis.