

# The del/del genotype of the nuclear factor- $\kappa$ B -94ATTG polymorphism and its relation to aggressive periodontitis

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**Background and Objective:** Periodontitis is influenced by specific host-dependent immune responses. Periodontopathogens induce innate immune responses, amongst others, via toll-like receptor 2 (TLR2), resulting in activation of the nuclear transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B). The aim of this case-control study was to evaluate links between genetic variants of these genes and chronic/aggressive periodontitis in a multivariate model.

**Material and Methods:** A total of 141 patients with periodontitis (63 with chronic periodontitis and 78 with aggressive periodontitis) and 81 controls without periodontitis were included in the study. Polymorphisms in TLR2 (Arg677Trp, Arg753Gln) and in NF- $\kappa$ B (-94ins/delATTG) were determined by restriction fragment length polymorphism and fragment length analyses, respectively. Subgingival bacterial colonization was evaluated using a PCR/DNA probe test (micro-Ident<sup>®</sup>).

**Results:** Although there was no association of the TLR2 polymorphism Arg753Gln with periodontitis, heterozygous carriers (Arg/Gln) were at a higher risk for colonization with bacteria of the 'red complex' (corrected  $p$ -value = 0.042). The del/del genotype of the NF- $\kappa$ B polymorphism was associated with aggressive periodontitis considering age, gender, smoking and approximal plaque index as potential confounders (odds ratio = 2.81,  $p$  = 0.035, 95% confidence interval: 1.08–7.33). del/del carriers had a higher risk for subgingival colonization with *Aggregatibacter actinomycetemcomitans* (odds ratio = 2.36,  $p$  = 0.030, 95% confidence interval: 1.09–5.1; adjusted for age, gender, smoking and pocket depth<sub>bacteria</sub>).

**Conclusions:** The del/del genotype of NF- $\kappa$ B was shown to be associated with the occurrence of aggressive periodontitis.

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Periodontitis, a chronic polymicrobial infection, is characterized as an immune response produced by the host against oral microbes that destroys the supporting tissue of the teeth, eventu-

ally leading to periodontal pocket formation, alveolar bone resorption and tooth loss (1). Factors modulating an individual host's immune response play a crucial role in the progression of the

disease. Gram-negative bacteria possess cell-wall components that are known to influence the innate immune response (2). Inflammatory and tissue-destructive processes caused by

infection with bacterial periodontopathogens are induced via membrane-bound toll-like receptors (TLRs), including TLR2, leading to the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) from the family of nuclear transcription factors (3,4). Activated NF- $\kappa$ B plays a critical role in the inducible expression of genes (such as cytokines) involved in many biological processes, including infection, inflammation and repair (5).

In periodontitis, the NF- $\kappa$ B signaling pathway is tightly regulated by different factors, including RANKL, tumour necrosis factor- $\alpha$  and interleukin-1 $\beta$ , in order to maintain alveolar bone homeostasis (6). Infections with periodontopathogens, including *Aggregatibacter actinomycetemcomitans* (3), *Prevotella intermedia* (7) and bacteria of the 'red complex' [*Tannerella forsythia* (8,9), *Porphyromonas gingivalis* (10) and *Treponema denticola* (11,12)], are very likely to be responsible for inducing the immune response. It has been demonstrated that cell-wall components of *P. intermedia* and bacteria of the 'red complex' induce signal transduction via TLR2, whereas *A. actinomycetemcomitans* has the ability to stimulate both TLR2 and TLR4 (7,13). Increased expression of TLR2, which triggers the intracellular cascade resulting in NF- $\kappa$ B activation, has been found in the connective tissue of patients with periodontitis (14), as well as in inflamed oral epithelium (15). In *in vitro* studies of the oral epithelial cell line H400, the bacterial-induced up-regulation of the NF- $\kappa$ B pathway, including TLR2, was confirmed (16). Several genomic variants of TLRs have been identified. The impact of functional variants of TLR4 in periodontal pathogenesis have been intensively investigated, with varying results obtained (17–21).

Two functionally important polymorphisms, Arg753Gln and Arg677Trp, have been described for TLR2 (22,23). The mutant allele of the Arg677Trp polymorphism was shown to abolish the NF- $\kappa$ B activation as a response to bacterial cell-wall components in transient transfection assays (24). Gautam *et al.* proved the crucial importance of Arg753 for TLR2 signalling using alanine

scanning mutagenesis (25). Because of their functional importance, for both polymorphisms attempts were made to link them to inflammatory diseases, such as periodontitis (17,26–28). However, no genotype-dependent association has been reported for chronic or aggressive periodontitis, possibly because of the low prevalence of the mutant alleles of both polymorphisms.

Human NF- $\kappa$ B is a heterodimer encoded by two different genes: NFKB1 and NFKB2 (4). An insertion/deletion polymorphism was described in the promoter of the NFKB1 gene (-94ins/del ATTG), which influenced the transcriptional expression (29). In transient transfection experiments, Karban *et al.* showed that this polymorphism affects promoter activity, particularly due stimulation of the innate immune system by bacterial cell-wall components. The deletion allele was associated with significantly reduced promoter activity. In different clinical studies possible associations between this genomic variant and inflammatory diseases, including ulcerative colitis (30), Crohn's disease (31), psoriatic arthritis (32), ankylosing spondylitis (33) and cancer (34,35), were investigated. However, no clinical studies have yet been published that evaluate the importance of this polymorphism in the development of periodontitis.

On the basis of the clinical data currently available, functionally important TLR2 and NF- $\kappa$ B polymorphisms may therefore have an impact on the composition of subgingival plaque and/or the inflammatory response to periodontopathogens contributing to the development of periodontitis. However, little is known about the complex interaction of the genetic background of TLR2 and NF- $\kappa$ B and clinical markers of periodontitis. Therefore, we carried out a clinical study to evaluate the effect of these polymorphisms in aggressive and chronic periodontitis, as well as in association with key periodontal bacteria in bivariate and multivariate models, considering established confounders for periodontitis, such as age, gender, smoking and the plaque index.

## Material and methods

### Study population and clinical investigations

The study was performed at the Department of Operative Dentistry and Periodontology of the Martin-Luther-University Halle-Wittenberg, from March 2005 to October 2008. Two-hundred and twenty two unrelated persons of the same Caucasian origin from Central Germany were recruited consecutively into our study consecutively. The groups of patients ( $n = 141$ ) comprised 78 patients with generalized aggressive periodontitis and 63 patients with generalized chronic periodontitis. The control group included 81 periodontitis-free participants (demographic data are given in Table 1).

All patients and controls were assessed in accordance with the classification system of periodontal diseases (1). Inclusion and exclusion criteria for all participants have been previously described in detail (36). In brief, patients were classified as having generalized chronic periodontitis if the showed attachment loss with a minimum pocket depth of 4 mm in at least 30% of their teeth. The degree of attachment loss was consistent with the presence of calculus. More horizontal than vertical approximal bone loss was visible in the radiographs. Patients were classified as having generalized aggressive periodontitis only if there was evidence (from dental history and/or radiographs) that the onset of the disease occurred before the patient reached 35 years of age; these patients had clinical attachment loss of  $\geq 4$  mm in at least 30% of their teeth. In order to exclude localized aggressive periodontitis, at least three of the affected teeth had not to be first molars or incisors. In contrast to chronic periodontitis, the severity of attachment loss in patients with aggressive periodontitis was inconsistent with the amount of calculus, and more vertical than horizontal approximal bone loss was visible in the radiographs. Periodontitis-free individuals were included if they were at least 30 years old and had no attachment loss [probing

Table 1. Clinical characteristics of the groups of patients and of the no-periodontitis controls

Variable	Aggressive periodontitis (n = 78)	Chronic periodontitis (n = 63)	No-periodontitis controls (n = 81)
Demographic parameters			
Average age (years)	40.6 ± 9.8	48.6 ± 9.6	46.6 ± 10.9
Female (%)	61.5	65.1	51.9
Current smoker (%)	34.6	22.2	22.2
Past smokers (%)	12.8	14.3	13.6
Clinical parameters			
Approximal plaque index (%)	52.3 ± 28.5	60.5 ± 25.6	47.5 ± 21.5
Bleeding on probing (%)	78.1 ± 22.7	69.5 ± 24.9	45.7 ± 23.7
Clinical probing depth (pocket depth in mm)	5.7 ± 1.4	5.2 ± 1.1	2.6 ± 0.8
Clinical attachment loss (in mm)	6.5 ± 1.5	5.9 ± 1.4	3 ± 0.8
Pocket depth <sub>bacteria</sub> (mm)	7.5 ± 1.6	6.8 ± 1.5	3.1 ± 0.4
Clinical attachment loss <sub>bacteria</sub> (mm)	8.4 ± 1.8	7.5 ± 1.8	3.3 ± 0.5
Individual occurrence of periodontal bacteria in subgingival pockets			
<i>Aggregatibacter actinomycetemcomitans</i> (%)	41.0	31.7	19.8
<i>Porphyromonas gingivalis</i> (%)	78.2	87.3	23.5
<i>Prevotella intermedia</i> (%)	64.1	61.9	33.3
<i>Tannerella forsythia</i> (%)	85.9	96.8	69.1
<i>Treponema denticola</i> (%)	87.2	98.4	64.2
Red complex <i>P.g.</i> + <i>T.f.</i> + <i>T.d.</i> (%)	71.8	82.5	23.5

*P.g.*, *Porphyromonas gingivalis*; *T.d.* *Treponema denticola*; *T.f.*, *Tannerella forsythia*.

Clinical attachment loss<sub>bacteria</sub>, clinical attachment loss at microbiological test site; Pocket depth<sub>bacteria</sub>, pocket depth at microbiological test site.

depth ≤ 3.5 mm, no gingival recession caused by periodontitis (37)]. Clinical attachment loss of > 3.5 mm as a consequence of traumatic toothbrushing or overhanging dental restorations was not considered as periodontitis. Although a clinically definite correlation of traumatic toothbrushing and attachment loss could be determined, a strict relationship between dental filling and ensuing periodontitis could not be assumed. It could not be completely excluded that the periodontal lesion existed before the dental filling was made. However, the strictly localized co-existence of periodontitis and an overhanging filling in an otherwise healthy periodontium is probably induced by the dental restoration.

In general, we excluded pregnant women and subjects who had a drug-induced gingival hyperplasia or who had received antibiotics in the 6 months before the start of the study. Subjects reporting chronic use of anti-inflammatory drugs and with a history of diseases associated with periodontitis were also excluded. The clinical assessment included determination

of the approximal plaque index, bleeding on probing, pocket depth and clinical attachment loss.

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

#### Molecular assessment of periodontopathic bacteria

For each proband the mean pocket depth and clinical attachment loss of these test sites (pocket depth<sub>bacteria</sub>, pocket depth at microbiological test site, clinical attachment loss<sub>bacteria</sub>, clinical attachment loss at microbiological test site) were recorded. Subgingival plaque samples were harvested before subgingival scaling was carried out. The plaque samples were taken from the deepest pocket of each quadrant by the insertion of a sterile paper point for 30 s. All samples of each individual were pooled in one tube. Prepa-

ration of bacterial DNA was carried out using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For specific amplification of *A. actinomycetemcomitans*, *P. intermedia* and bacteria of the 'red complex' (*P. gingivalis*, *T. forsythia* and *T. denticola*), the micro-Ident<sup>®</sup> test (Hain Lifescience, Nehren, Germany), based on alkaline phosphatase-mediated staining, was used. PCR amplification was performed (5 min at 95°C; 10 cycles of 30 s at 95°C, 2 min at 58°C, 40 s at 70°C; 20 cycles of 25 s at 95°C, 40 s at 53°C, 40 s at 70°C; and 8 min at 70°C) in a personal cyler (Biometra, Göttingen, Germany). The PCR products were hybridized to a strip containing DNA sequences of each bacterium as well as positive controls for amplification and hybridization. The presence of the bacteria was determined visually by means of coloured bands. Two positive controls for the amplification reaction and for the conjugate were included in the test.

#### Genetic studies

For genetic investigations, fresh venous EDTA-anticoagulated blood was obtained from the test subjects. Preparation of genomic DNA was carried out using a QIAamp<sup>®</sup> blood extraction kit (Qiagen) in accordance with the manufacturer's instructions.

For genotyping the polymorphisms Arg753Gln and Arg677Trp in the TLR2 gene, restriction fragment length polymorphism analysis was applied. Because of the use of specific primers (Arg753Gln: forward primer, 3'-cat tcc cca gcg ctt ctg caa gct cc-5' and reverse primer, 3'-gga acc tag gac ttg atc gca gct c-5'; Arg677Trp: forward primer, 3'-ccc ctt caa gtt gtg gct tca taa g-5' and reverse primer, 3'-agt cca gtt cat act tgc acc ac-5') recognition sites for restriction enzymes (*MspI* in Arg753Gln and *MwoI* in Arg677Trp) were introduced as previously described (17). For every PCR amplification, 25 µL of a master-mix containing 1 U of Taq polymerase (Invitex, Berlin, Germany), 50 ng of genomic DNA, 1% formamide and PCR reaction buffer was added. PCR amplification was performed in an

Eppendorf Mastercycler Gradient (2 min at 94°C; 12 cycles of 30 s at 92°C, 30 s at 63°C (Arg753Trp) or 65°C (Arg677Trp), 30 s at 72°C; 22 cycles of 30 s at 92°C, 30 s at 58°C (Arg753Trp) or 60°C (Arg677Trp), 30 s at 72°C, and a final extension for 10 min at 72°C, ending with cooling up to 4°C). Restriction fragment analysis was performed at 37°C, overnight, using 10 U of the appropriate restriction endonuclease (New England Biolabs, Frankfurt, Germany). The fragments were evaluated visually after separation in a 2% agarose gel stained with ethidium bromide.

Genotyping of the -94 ins/del ATTG polymorphism in the NF- $\kappa$ B gene was performed using fragment length analysis (forward primer 5'-tgg acc gca tga ctc tat ca-3'; reverse primer 5'-gaa tcc caa ggg ctg ga-3'). PCR amplification was performed in an Eppendorf Mastercycler Gradient (2 min at 95°C; 12 cycles of 30 s at 92°C, 30 s at 55°C, 30 s at 72°C; 21 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C, 5 min at 72°C, 10°C hold). The DNA fragments were separated by electrophoresis through a polyacrylamide (PAA) gel (PAA gel: Crosslinker concentration = 10.4%, Total acrylamide-bisacrylamide monomer concentration = 3.7%) and visualized by silver staining.

All sequence data were identical to the sequence of the human TLR2 gene (GenBank accession number NM 003264) and the NF- $\kappa$ B gene (GenBank accession number AF 213884S1).

#### Statistical evaluation

Statistical analyses were carried out using the program spss 15.0 (SPSS Inc., Chicago, IL, USA);  $p$ -values of  $\leq 0.05$  were considered 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 the  $n$ -value was  $< 5$ , Fisher's exact test was performed. Metric parameters are presented as mean  $\pm$  standard error. The data were analysed using the

Kolmogorov-Smirnov test (test of normal distribution). For the statistical evaluation the Student's  $t$ -test or one-way analysis of variance (normally distributed values) and Mann-Whitney  $U$ -test or Kruskal-Wallis test (values not distributed normally) were used. Binary logistic regression analysis was used for investigating the impact of polymorphic variants on the development of aggressive periodontitis and subgingival colonization of periodontopathogens considering established confounders. Interaction of polymorphic variants and clinical markers of periodontitis, as well as smoking, were evaluated on additive scales by estimating the interaction contrast ratio (38). The statistical significance of the interaction effect was confirmed if the interaction contrast ratio 95% confidence interval excluded zero.

## Results

### Clinical assessment

All individuals involved in the study were evaluated according to their age, gender and smoking status. When comparing the groups of patients with the periodontitis-free healthy controls, no statistically significant differences in gender and smoking status could be detected. In accordance with the inclusion criteria, the mean age of patients in the aggressive periodontitis group was significantly lower.

In comparison with the control probands, the clinical parameters of periodontitis such as approximal plaque index, bleeding on probing, pocket depth and clinical attachment loss were significantly elevated in the two groups of patients. However no statistical difference was obtained comparing the approximal plaque index in patients with aggressive periodontitis and periodontitis-free controls. In both groups of patients there was an increase in the occurrence of bacteria. However, there was no significant difference with respect to *A. actinomycetemcomitans* between patients with chronic periodontitis and the control group. Clinical and demographic data are given in Table 1.

### Genetic investigations

*Bivariate analyses* — No homozygote or heterozygote mutation carrier of the Arg677Trp polymorphism in TLR2 gene was identified within the study group. The genotype distributions of the polymorphisms (NF- $\kappa$ B: -94 ins/del ATTG; TLR2: Arg753Gln) were in Hardy-Weinberg equilibrium.

Evaluation of the Arg753Gln polymorphism in the TLR2 gene (Table 2), identified a distinct, but not significant, increase in the number of carriers of this heterozygous mutation in the group of patients (aggressive periodontitis + chronic periodontitis) compared with periodontal healthy individuals (OR: 2.7, 95% CI: 0.58–12.99). After subdividing the patients according to their clinical status (aggressive periodontitis and chronic periodontitis group) this tendency remained (aggressive periodontitis: odds ratio, 2.7; 95% CI, 0.52–14.49) (chronic periodontitis: OR, 2.7; 95% CI, 0.48–15.38).

Investigating the influence of the NF- $\kappa$ B -94 ins/del ATTG-polymorphism revealed an association of the del/del with aggressive periodontitis ( $p = 0.05$ ). Considering an ins-dominant genetic model, this association became statistically significant (odds ratio: 2.74, 95% confidence interval: 1.1–6.75, Yates-corrected  $p = 0.042$ ). Possible links between genetic variants and microbiological findings [e.g. the occurrence of periodontal bacteria, including *A. actinomycetemcomitans*, *P. intermedia* and bacteria of the 'red complex' (*P. gingivalis*, *T. forsythensis* and *T. denticola*), in subgingival pockets] and clinical data (approximal plaque index, bleeding on probing, pocket depth and clinical attachment loss) were assessed in the entire study group.

Subgingival colonization with bacteria of the 'red complex' was over-represented in probands carrying the Arg/Gln genotype of the Arg753Gln polymorphism in TLR2 (Table 3, odds ratio: 8.17, 95% confidence interval: 1.03–65.0, Yates-corrected  $p = 0.042$ ). The del/del genotype of the -94 ins/del ATTG polymorphism in NF- $\kappa$ B was significantly associated with the subgingival occurrence of

Table 2. Genotype and allele distribution of single nucleotide polymorphisms in the nuclear factor-κB (NF-κB) gene (-94 ins/del ATTG) and in the toll-like receptor 2 (TLR2) gene (Arg753Gln) and their effect on the occurrence of aggressive periodontitis (AP) and chronic periodontitis (CP)

	All patients	AP	CP	No-periodontitis controls	AP + CP vs. controls <i>p</i> -value	AP vs. controls <i>p</i> -value	CP vs. controls <i>p</i> -value
NF-κB: -94 ins/del ATTG							
	( <i>n</i> = 141)	( <i>n</i> = 78)	( <i>n</i> = 63)	( <i>n</i> = 81)			
ins/ins (%)	36.1	35.9	36.5	34.6			
ins/del (%)	44	41.0	47.6	55.6			
del/del (%)	19.9	23.1	15.9	9.9	NS*	0.042*	NS*
ins (%)	58.2	56.4	60.3	62.3			
del (%)	41.8	43.6	39.7	37.7	NS	NS	NS
TLR2: Arg753Gln							
	( <i>n</i> = 139)	( <i>n</i> = 77)	( <i>n</i> = 62)	( <i>n</i> = 81)			
Arg/Arg (%)	93.5	93.5	93.5	97.5			
Arg/Gln (%)	6.5	6.5	6.5	2.5	NS	NS	NS
Arg (%)	96.8	96.8	96.8	98.8			
Gln (%)	3.2	3.2	3.2	1.2	NS	NS	NS

\*Significances were estimated considering an ins-dominant genetic model (ins/ins + ins/del vs. del/del), Yates-corrected *p*-value. NS, nonsignificant.

Table 3. Association of the individual nuclear factor-κB (NF-κB) (-94 ins/del ATTG) and toll-like receptor 2 (TLR2) (Arg753Gln) genotype distributions, and the subgingival occurrence of *Aggregatibacter actinomycetemcomitans*, and bacteria of the 'red complex' (*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*) in the whole study group

	<i>p</i> -value	Odds ratio (95% CI)
<i>A. actinomycetemcomitans</i> (%)		
NF-κB: -94 ins/del ATTG		
ins/ins + ins/del ( <i>n</i> = 186)	27.4	0.031*
del/del ( <i>n</i> = 36)	47.2	2.37 (1.14–4.91)
Bacteria of the 'red complex' (%)		
TLR2: Arg753Gln		
Arg/Arg ( <i>n</i> = 209)	55.0	0.026*
Arg/Gln ( <i>n</i> = 11)	90.9	8.17 (1.03–65)

\*Yates corrected *p*-value.  
CI, confidence interval.

*A. actinomycetemcomitans* (Table 3, odds ratio: 2.37, 95% confidence interval: 1.14–4.91, Yates-corrected *p* = 0.031).

No other significant links between the investigated gene polymorphisms and subgingival bacterial colonization or clinical parameters of periodontitis were found in the entire study group. In addition, no dependence of

other factors known to affect periodontitis on the genetic background of NF-κB and TLR2 (including age, gender and smoking status) could be determined.

*Multivariate analyses (binary logistic regression analysis)* — In a first model the risk of carriers of the homozygote del/del genotype of the NF-κB gene

suffering from aggressive periodontitis was assessed, considering age, gender, smoking and approximal plaque index as confounding factors (Table 4). In this multivariate model, the del/del genotype has been found as a significant predictor for aggressive periodontitis (odds ratio = 2.81, *p* = 0.035, 95% confidence interval: 1.08–7.33).

In a second evaluation, the influence of genetic constellations on the subgingival colonization with *A. actinomycetemcomitans* (NF-κB genotype del/del) and bacteria of the 'red complex' (TLR2 genotype Arg/Gln) were assessed with respect to the possible cofounders age, gender, smoking and probing depth at microbiological test sites. In the corresponding statistical model, the del/del genotype of NF-κB has been verified as an independent risk factor for the occurrence of *A. actinomycetemcomitans* (Table 5; odds ratio = 2.36, 95% confidence interval: 1.09–5.1, *p* = 0.030). Possible interactions of the del/del genotype

Table 4. Binary logistic regression model investigating the adjusted\* odds ratio of the nuclear factor-κB (NF-κB) genotype del/del for the occurrence of aggressive periodontitis

	Regression coefficient	SE	<i>p</i> -value	Odds ratio	95% CI	
					Lower	Upper
NF-κB genotype del/del	1.033	0.49	0.035	2.81	1.08	7.33

\*Adjusted for the potential confounders age, gender, smoking and approximal plaque index. CI, confidence interval; SE, standard error.

Table 5. Binary logistic regression model evaluating the adjusted\* odds ratio of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) genotype del/del for the occurrence of *Aggregatibacter actinomycetemcomitans*

	Regression coefficient	SE	<i>p</i> -value	Odds ratio	95% CI	
					Lower	Upper
NF- $\kappa$ B genotype del/del	0.857	0.39	0.030	2.36	1.09	5.1

\*Adjusted for the potential confounders age, gender, smoking and probing depth at microbiological test sites (pocket depth<sub>bacteria</sub>). CI, confidence interval; SE, standard error.

and clinical markers of periodontitis, as well as smoking, were investigated by estimating the interaction contrast ratio. However, no statistically significant interaction could be found.

There was no proof of a statistically significant influence of the Arg/Gln genotype of the TLR2 polymorphism Arg753Gln on subgingival colonization with bacteria of the 'red complex' (odds ratio: 6.54, 95% confidence interval: 0.75–58.82). The probing depth at microbiological test sites ( $p < 0.001$ , odds ratio = 1.68, 95% confidence interval: 1.43–1.96) and age of the subject ( $p = 0.002$ , odds ratio = 1.05, 95% confidence interval: 1.02–1.08) were the only significant predictors for the occurrence of these bacteria in this model (data not shown).

## Discussion

It has been established that the pathogenesis of periodontitis is initiated and maintained by subgingival bacterial infection. Furthermore, the impact of individual genetic variation on the development of both aggressive and chronic periodontitis has been proven in previous clinical studies (39,40). Thus, genes involved in the individual host's immune response are considered as susceptibility genes for periodontitis (40). In this clinical case-control study, genomic variants of genes coding for important modulators of the signal transduction cascade, leading to transcriptional activation of the inflammatory target genes TLR2 (Arg677Trp, Arg753Gln) and NF- $\kappa$ B (-94ins/del ATTG), were analyzed.

### Clinical investigations

Patients were diagnosed on the basis of clinical, radiographic and historical findings. Demographic data of

the proband groups were evaluated. Patients suffering from aggressive periodontitis were significantly younger than periodontitis-free controls. As aggressive periodontitis is expected to become clinically apparent at a younger age (before 30 years of age in most cases), control probands who were older than 30 years of age represent a more suitable comparison group than younger controls for investigations of periodontal risk factors (41,42). Following the evaluation of clinical features of periodontitis, including approximal plaque index, bleeding on probing, pocket depth, pocket depth<sub>bacteria</sub>, clinical attachment loss and clinical attachment loss<sub>bacteria</sub>, as well as subgingival bacterial colonization, the expected presumptions were confirmed (21,43,44).

### Genetic investigations

In the present study the impact of polymorphisms in TLR2 and NF- $\kappa$ B on the occurrence of aggressive and chronic periodontitis was analyzed. Moreover, the effect of these genetic variants on the subgingival colonization of five species of periodontopathic bacteria was investigated. In clinical studies carried out previously, the importance of individual genetic backgrounds on the occurrence of subgingival periodontopathogens was found for polymorphisms in interleukin-1 (45,46), tumour necrosis factor- $\alpha$  (47) and CD14 (21).

As periodontitis and its clinical markers are affected by a variety of other factors, including gender (42), smoking (44,48,49) and age (41,42), a more complex model is required to evaluate the true impact of genetic variations.

Functionally important polymorphisms in TLR2 (Arg677Trp, Arg753Gln) may impair the immune

response to periodontopathogens via modified NF- $\kappa$ B activation, leading to variation in gene-expression patterns. Consequently, they could have an influence on the initiation and progression of periodontitis. However, our investigations regarding the Arg677Trp polymorphism revealed no carrier of the mutant 677Trp allele in the entire study group. This finding confirms previously published observations of clinical studies on periodontitis (17,26–28). Nevertheless, this genomic variant has been reported to be associated with lepromatous leprosy (23) possibly because of an impaired immune response to *Mycobacterium leprae* (24). With respect to these results, it can be speculated that this polymorphism indeed has functional consequences for the proper detection of pathogens but plays no crucial role in the recognition of cell-wall components of periodontopathogens.

In the present study, the Arg753Gln polymorphism had a distinct, but no statistically significant, influence on the occurrence of periodontal diseases (aggressive and/or chronic), in accordance with previously published data (17,26–28). However, because the occurrence of the mutant genotype/allele is a relatively rare event, the study may be underpowered in this respect. The frequencies found for the mutant allele ranged from 2.5% (controls) to 6.5% (patients with periodontitis) and were in agreement with the results from previous studies (17,26–28). As varying frequencies for the heterozygous genotype are described for healthy controls in different clinical studies (17,22,50–52) we could not exclude an ethnic-specific effect of the genotype. For the first time, a significant association between this polymorphism and the occurrence of periodontopathogens of the 'red

complex' has been demonstrated. Furthermore, carriers of the mutant allele were more frequently affected with *A. actinomycetemcomitans* and *P. intermedia* (not significant). Because Arg753 has been shown to be crucial for TLR2 signalling (25), it is possible that the immune response to periodontopathogens associated with the recognition of bacterial components may be impaired as a result of this mutant allele. However, when considering established confounders of periodontitis, including gender, age, smoking status and probing depth at a microbiological test site, this association could not be confirmed. This finding suggests that other predictors may be more important for determining the occurrence of periodontal diseases than the genetic background of TLR2.

Activated NF- $\kappa$ B is responsible for the regulation of many genes involved in immune response, cell adhesion, differentiation, proliferation, angiogenesis and apoptosis. This study revealed, for the first time, a significant association of the homozygous mutation genotype del/del with the occurrence of aggressive periodontitis in a cohort of German Caucasians. In multivariate analysis, the impact of the del/del genotype could be emphasized after adjustment for age, approximal plaque index, gender and smoking status as confounders of aggressive periodontitis. For patients suffering from chronic periodontitis, a similar, but not significant, influence of the del/del genotype was apparent. The del allele was associated with decreased promoter activity of NF- $\kappa$ B (29). Furthermore, the induction of NF- $\kappa$ B activity as a result of stimulation with bacteria, including periodontopathogens (16), has been reported to play a crucial role in host defence. In accordance with this notion, a del/del-dependent impaired immune response to periodontopathogens could be assumed. And, indeed, when investigating putative correlations of the genotype with subgingival bacterial colonization, a significant association of the del/del genotype and the occurrence of *A. actinomycetemcomitans* could be shown. Even after adjustment for age, gender, smoking and probing

depth at the microbiological test site, the presence of *A. actinomycetemcomitans* was significantly associated with the del/del genotype.

#### Limitations of the study

The case-control study presented above was conducted to establish assumptions of possible associations between genomic variants and periodontitis and periodontopathogens. However, it was not possible to verify these assumptions using the present study design. The sample size of the study was relatively small and therefore the risk of type 2 error was considerable, especially when interpreting the effects of interactions, as the statistical power to detect interaction effects is generally lower than the ability to detect the main effects.

The control group, representing periodontitis-free controls [defined by Ref. (37)], was recruited from the dental department of the Martin-Luther-University Halle-Wittenberg. For these periodontitis-free controls, further dental disease could not be excluded, which may potentially interfere with the susceptibility to periodontitis. Accordingly, the possible selection bias may result in an underestimation of the genotype-dependent effect.

As the data presented were collected from stringently selected patients and periodontitis-free controls from a distinct geographical region, the results can be considered to be applicable for Caucasians of Central Germany only and must therefore be interpreted with necessary caution. It is not appropriate to extrapolate the results to the general population.

#### In conclusion

Despite the limitations of this study, our results demonstrate a significant impact of the genetic background of two important modulators (TLR2 and NF- $\kappa$ B) of the immune response to periodontopathogens on the presence of periodontitis, as well as on the subgingival colonization of bacteria. The results of this clinical study emphasize the importance of the del/del genotype of the NF- $\kappa$ B -94ins/del ATTG polymor-

phism as a risk indicator for aggressive periodontitis in this German cohort.

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