

# Genetic variants in the coding region of TGFb1 and the subgingival occurrence of periodontopathogens

**Language:** English

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## Introduction

Periodontitis is characterized as a chronic inflammatory disease induced by periodontopathogens. TGF- $\beta$ 1 is a pleiotropic cytokine that exerts its effects on bone and connective tissue metabolism which are of great importance in periodontal disease.

Functional important polymorphisms are described for TGF- $\beta$ 1:

(L10P) associated with higher circulating levels and secretion of TGF- $\beta$ 1 Yokota et al.2000; Dunning et al., 2003  
 (R25P) associated with interindividual variation in TGF- $\beta$ 1 Awad et al. 1998

Signalling pathways mediated by TGF- $\beta$ 1

TGF- $\beta$ 1 as a proinflammatory cytokine plays an important role in mediating pathogen dependent regulation of the immune system. TGF- $\beta$ 1 has been shown to be downregulated in active periodontal lesions showing clinical evidence of periodontal tissue destruction. Downregulation of TGF- $\beta$ 1 is supposed to lead to the overexpression of cytokines, including e.g. RANKL, promoting the pathogenesis of periodontitis.

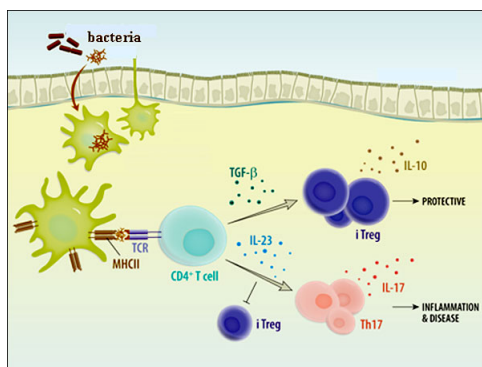


Fig. 1: Signalling pathways mediated by TGF- $\beta$ 1 According to [www.rndsystems.com/dam\\_public/6103.png](http://www.rndsystems.com/dam_public/6103.png)

## Aim of the study

In the present study possible associations were investigated between the genetic variants of TGF- $\beta$ 1 and chronic/aggressive periodontitis and its clinical features, including smoking status, plaque (API) and bleeding index (BOP), pocket depth (PD), clinical attachment loss (CAL) and subgingival bacterial colonization.

## Material and Methods

## **Inclusion criteria of probands**

### Generalized aggressive periodontitis (AP) n=81:

clinical manifestation before 35th year of life attachment loss in at least 30% of the teeth with a minimum pocket depth of 4 mm > 3 affected teeth had to be no first molars or incisors  
severity of attachment loss was inconsistent to the amount of mineralized plaque  
more vertical than horizontal approximal bone loss was visible in the radiographs

### Generalized chronic periodontitis (CP) n=68:

attachment loss in at least 30% of the teeth with a minimum pocket depth of 4 mm  
The amount of the attachment loss was consistent with the presence of mineralized plaque  
More horizontal than vertical approximal bone loss was visible in the radiographs.

### Periodontitisfree controls n=82:

probing depth  $\leq$  3.5 mm, no gingival recession due to periodontitis Clinical attachment loss > 3.5mm as a consequence of traumatic tooth brushing, overhanging dental fillings, orthodontic therapy etc. was not considered as a case of periodontitis.

## **Genomic investigation**

### DNA-isolation from EDTA-blood

Preparation of genomic DNA from human venous EDTA-blood was carried out using the blood extraction kit (Quiagen).  
200 $\mu$ l EDTA-blood and 20  $\mu$ l protease were mixed in a 1,5 ml tube.  
After adding of 200  $\mu$ l denaturation buffer AL and pulse-vortexing for 15 sec the samples were incubated at 56°C for 10 min.  
200  $\mu$ l of ethanol was added to the samples, vortexed and the samples were applied to a QIAamp Spin Column where the DNA is bound.  
After two washing steps (buffer AW1 and AW2) the DNA bound to the column is dried by centrifugation.  
200  $\mu$ l distilled water is added to the samples, incubated at room temperature for 5 min and then centrifuged. The solved DNA is now in the filtrate.  
Long-term storage of DNA is possible at -20°C.

### Haplotype specific PCR of TGF- $\beta$ 1

The detection of genotypes and haplotypes of TGF- $\beta$ 1 SNPs (L10P and R25P) was carried out using the CYTOKINE Genotyping array CTS-PCR-SSP Tray kit of the Collaborative Transplant Study, Department of Transplantation Immunology of the University Clinic of Heidelberg.  
For every PCR a fragment of 440bp of the human CRP gene was coamplified as a positive control.  
The PCRs were performed using sequence specific primers for detection of possible haplotypes pipetted and lyophilized in thin-walled plastic 96-well PCR trays.  
For every PCR 10 $\mu$ l of a Mastermix containing 1U Taq-Polymerase (Invitex), 100ng genomic DNA, 5% glycerol, and PCR reaction buffer was added.  
PCR-program (2 min 94°C; 10 cycles: 15 sec 94°C, 1 min 64°C; 20 cycles: 15 sec 94°C, 50 sec 61°C, 30 sec 72°C)  
After cycling was completed, the PCR products were loaded onto a 2% agarosegel for electrophoresis.  
After electrophoresis, the ethidium bromide stained gel is photographed and interpreted.

## **Evaluation of periodontopathic bacteria in subgingival pockets**

Paper points for collection of subgingival samples were used to bind periodontopathogens of the deepest pocket of each quadrant.

### DNA-isolation

Preparation of bacterial DNA was carried out using the QIAamp DNA Mini Kit (Quiagen).  
The paper points were incubated with 180  $\mu$ l ATL-buffer and 20  $\mu$ l proteinase K and incubated at 70°C for 10 min.  
200  $\mu$ l buffer AI was added and the mixture was incubated at 96°C for 5 min.  
The mixture (without paper points) was applied to a QIAamp Spin Column and washed twice with buffer AW1 and AW2.  
The DNA was solved in 400  $\mu$ l AE-buffer and stored at -20°C.

### Multiplex-PCR

For specific amplification of Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Treponema denticola the micro-Ident® test of HAIN-Diagnostik based on alkaline phosphatase mediated staining reaction was used.  
Mastermix provided in the micro-Ident® test (containing buffer, biotinylated primer, DNA for positive control), 2U Taq-polymerase (Eppendorf), and 5  $\mu$ l of isolated bacterial DNA were mixed.  
PCR was performed (5 min 95°C; 10 cycles: 30 sec 95°C, 2 min 58°C; 20 cycles: 25 sec 95°C, 40 sec 53°C, 40 sec 70°C; 8 min 70°C)  
The quality of PCR product was checked by agarosegelelectrophoresis.

### Bacteria specific hybridization

20  $\mu$ l of the PCR product were mixed with 20  $\mu$ l of the denaturation solution in the well of the tray and incubated at room temperature for 5 min.  
1 ml prewarmed (45°C) hybridization buffer was added to the sample and a strip (hybridized with DNA sequences of each bacteria as well as a positive control) was placed in the well of the tray.  
The tray was incubated at 45°C for 30 min in a shaking water bath.  
1 ml of stringent wash solution was added and incubated at 45°C for 15 min.  
The strip was washed once with 1 ml rinse solution for 1 min and 1 ml of conjugate solution was added (room temperature for 30 min).  
After washing 1 ml of substrate solution was added.  
The occurrence of bacteria was evaluated visually by means of colored bands.  
Two positive controls for amplification reaction and for conjugate were included in the test.

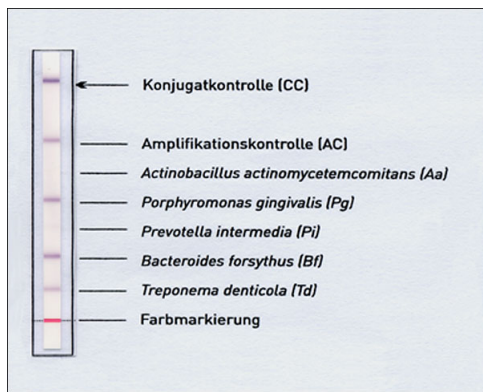


Fig. 2: Evaluation of periodontopathic bacteria in subgingival pockets according to HAIN-Diagnostic

## Results

	<b>Chronic periodontitis (CP)</b> <b>n=68</b>	<b>Aggressive periodontitis (AP)</b> <b>n=81</b>	<b>healthy controls</b> <b>n=82</b>	<b>p values vs. controls</b>	
				<b>CP</b>	<b>AP</b>
Mean age (years)	48.9±9.6	40±9.5	46.6±10.7	n.s.	< 0.001
Gender (% female)	64.2	63	53.7	n.s.	n.s.
Smoking (%)	25	34.6	21.2	n.s.	n.s.
Approximal plaque index (%)	61.8±25.5	52.8±28.5	47.3±21.4	< 0.001	n.s.
Bleeding on probing (%)	70.3±24.6	78±23.2	46±23.9	< 0.001	< 0.001
Pocket depth (mm)	5.2±1.2	5.7±1.4	2.6±0.7	< 0.001	< 0.001
Pocket depth on microbial test site (mm)	6.9±1.6	7.5±1.6	3.1±0.4	< 0.001	< 0.001
Clinical attachment loss in general (mm)	6±1.5	6.5±1.5	3.0±0.8	< 0.001	< 0.001
Clinical attachment loss on microbial test site (mm)	7.6±1.9	8.4±1.8	3.3±0.5	< 0.001	< 0.001

Tab. 1: Clinical characterization of the patient groups

No significant differences between the patient groups and the healthy control group could be proven investigating gender, smoking status, and age. Sole exception was the age of the patients suffering from aggressive periodontitis because of the young age of onset of disease. As expected, both patient groups showed significant more severe clinical symptoms compared to the control group.

Aggregatibacter actinomycetemcomitans (%)	30.6	47.2	22.2	n.s.	0.001
Porphyromonas gingivalis (%)	86.6	76.5	23.5	< 0.001	< 0.001
Prevotella intermedia (%)	61.8	61.7	32.9	< 0.001	< 0.001
Tannerella forsythia (%)	97.1	86.4	68.2	< 0.001	0.005
Treponema denticola (%)	98.5	85.2	64.7	< 0.001	0.002
Pg, Td, Tf (%)	82.4	71.3	23.5	< 0.001	< 0.001

Tab. 2: Microbiological characterization of the patient groups

As expected, both patient groups showed distinct and mostly significant increase in the occurrence of periodontopathic bacteria. Interestingly, no significant difference in the subgingival colonization with Aa could be shown for patients suffering from CP.

## Genetic evaluation

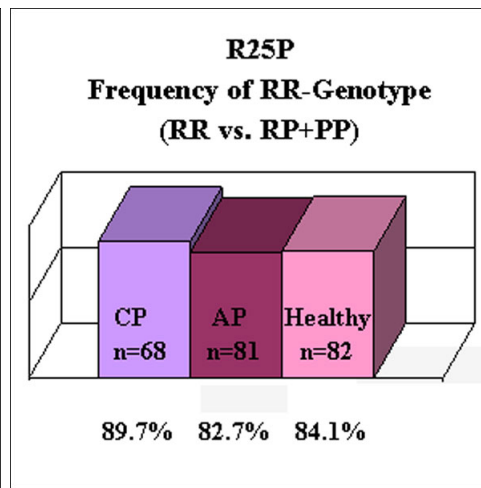
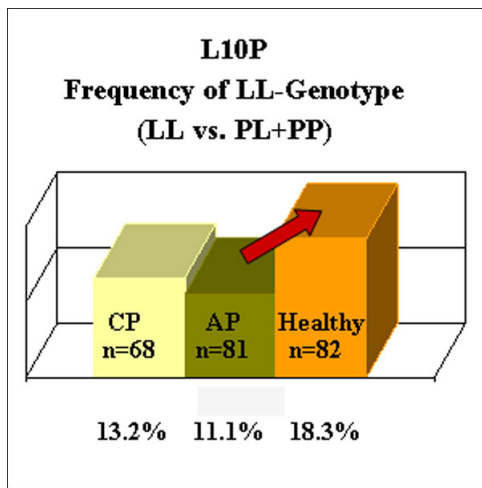


Fig. 3a-3b: TGF- $\beta$ 1 genotype/haplotype frequencies and the occurrence of periodontitis

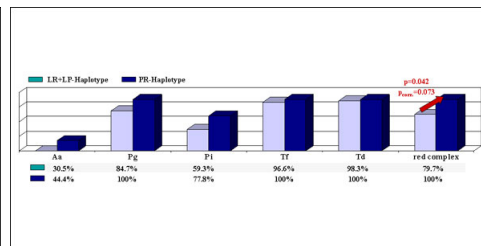
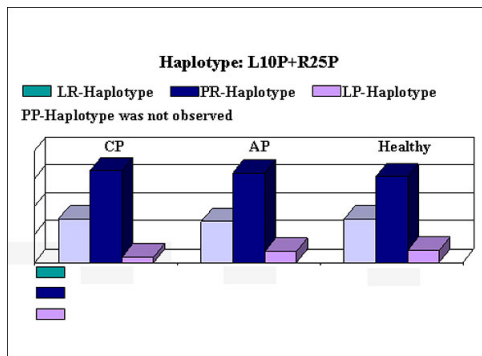


Fig. 3c: TGF- $\beta$ 1 genotype/haplotype frequencies and the occurrence of periodontitis

Fig. 4: TGF- $\beta$ 1 haplotype frequencies and the subgingival colonization with periodontopathogens in patients suffering from chronic periodontitis

## Conclusions

Hardy-Weinberg criteria were fulfilled for both TGF- $\beta$ 1 SNPs L10P and R25P.

No significant association of the genetic background of TGF- $\beta$ 1 including genotype and haplotype analyses and the occurrence of either chronic or aggressive periodontitis could be proven. A trend for a lower occurrence of the LL-genotype in patients suffering from aggressive periodontitis could be demonstrated (n.s.). Furthermore, a tendency towards a lower occurrence of LR-Haplotype among patients with chronic periodontitis could be described (n.s.).

Among patients with chronic periodontitis bacteria of the red complex (P.g., T.f., T.d.) occurred less frequently in carriers of the PR-haplotype ( $p=0.042$ ). After Yates correction the association become not significant ( $p_{corr}=0.073$ ).

However, in binary logistic regression analyses the SNPs L10P and R25P and haplotypes could not be proven as independent risk factors for bacterial colonization considering age, gender, smoking and API as cofounders.

This Poster was submitted by *Dr. Susanne Schulz*.

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# Genetic variants in the coding region of TGF-β1 and the subgingival occurrence of periodontopathogens



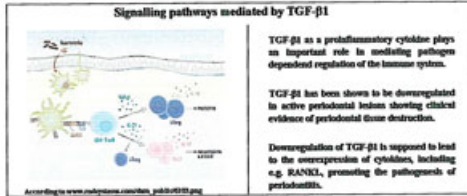
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## Introduction

Periodontitis is characterized as a chronic inflammatory disease induced by periodontopathogens.  
 TGF-β1 is a pleiotropic cytokine that exerts its effects on bone and connective tissue metabolism which are of great importance in periodontal disease.

Functional important polymorphisms are described for TGF-β1:  
 (L18F) associated with higher circulating levels and secretion of TGF-β1 Yokota et al., 2000;  
 Dawson et al., 2003  
 (G23F) associated with interstitial fibrosis in TGF-β1 Awwad et al., 1998

**Aims of the study:**  
 In the present study possible associations were investigated between the genetic variants of TGF-β1 and clinical/aggressive periodontitis and its clinical features, including smoking status, plaque (AP) and bleeding indices (BOP), pocket depth (PD), clinical attachment loss (CAL) and subgingival bacterial colonization.



TGF-β1 as a proinflammatory cytokine plays an important role in mediating pathogen dependent regulation of the immune system.

TGF-β1 has been shown to be downregulated in active periodontal lesions showing clinical evidence of periodontal tissue destruction.

Downregulation of TGF-β1 is supposed to lead to the overexpression of cytokines, including e.g. RANKL, promoting the pathogenesis of periodontitis.

## Material and Methods

### Inclusion criteria of probands

**Generalized aggressive periodontitis (AP):**  
 n=61  
 clinical manifestation before 35th year of life  
 attachment loss in at least 20% of the teeth with a minimum pocket depth of 4 mm  
 > 3 affected teeth before the first radiolucid or radiopaque  
 severity of attachment loss was determined by the amount of interdental plaque  
 were verified from histological specimens from four sites within the subgingival

**Generalized chronic periodontitis (CP):**  
 n=68  
 attachment loss in at least 20% of the teeth with a minimum pocket depth of 4 mm  
 The amount of the attachment loss was considered with the presence of interstitial plaque  
 mass histological from verified aggressive bone loss was verified in the radiographs

**Periodontitis-free control:**  
 n=62  
 attachment loss < 1.5 mm, no gingival recession due to periodontitis  
 Clinical attachment loss > 3 mm as a consequence of traumatic tooth brushing, occluding  
 dental filling, orthodontic therapy etc. was not considered as a case of periodontitis.

### Genomic investigations

**DNA isolation from EDTA blood**  
 Preparation of genomic DNA from human tissue EDTA blood was carried out using the blood extraction kit (Qiagen)  
 200 µl EDTA blood and 20 µl proteinase were added in a 1.5 ml tube.  
 After adding of 200 µl lysis buffer and 10 µl of proteinase the tubes were incubated at 50°C for 10 min  
 100 µl of ethanol was added to the sample, vortexed and the samples were applied to QIAamp Spin Column from the DNA is bound.  
 After two washing steps (Buffer AW1 and AW2) the DNA bound to the column is directly eluted.  
 200 µl distilled water is added to the sample, incubated at room temperature for 5 min and then centrifuged. The eluted DNA is now in the Eluate.  
 Long-term storage of DNA is possible at -20°C.

**Haplotype specific PCR of TGF-β1**  
 The detection of genotypes and haplotypes of TGF-β1 (509 G>A and 523F>C) was carried out using the CPTOWER Overlapping assay CPT PCR-SSP  
 The kit of the Collaborative Bioproducts, Department of Transplantation Immunology of the University Clinic of Heidelberg.  
 For every PCR a fragment of 440bp of the human CYP gene was amplified as a positive control.  
 The PCRs were performed using sequence specific primers for detection of possible haplotypes prepackaged and lyophilized in this method plastic 96-well PCR trays.

For every PCR 10 µl of a Mastermix containing 10 U Taq-Polymerase (Qiagen), 100 µg genomic DNA, 5% glycerol, and PCR reaction buffer was added.  
 PCR program (2 min 94°C, 10 cycles: 1 min 94°C, 30 sec 60°C, 30 sec 72°C, 30 sec 72°C)  
 After cycling was completed, the PCR products were loaded into a 96-well agarose gel for electrophoresis.  
 After electrophoresis, the ethidium bromide stained gel of 20 µl was photographed and interpreted.

### Evaluation of periodontopathic bacteria in subgingival pockets

**Subgingival sampling**  
 Paper points for collection of subgingival samples were used to find periodontopathogens of the deepest pocket of each tooth.

**DNA isolation**  
 Preparation of bacterial DNA was carried out using the QIAamp DNA Mini Kit (Qiagen).  
 The paper points were incubated with 100 µl ATL-buffer and 20 µl proteinase K and incubated at 56°C for 30 min.  
 100 µl lysis buffer AL was added and the mixture was incubated at 56°C for 3 min.  
 The mixture (without paper points) was applied to a QIAamp Spin Column and washed twice with buffer AW1 and AW2.  
 The DNA was eluted in 400 µl of ATL-buffer and stored at -20°C.

**Multiplex-PCR**  
 For specific amplification of Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia,  
 Treponema denticola the seven distinct sets of 2620 oligonucleotides based on distinct phylogenetic mediated cloning reactions was used.  
 Mastermix provided in the same kit used for sequencing buffer, hotstart polymerase (Qiagen), and  
 1 µl of isolated bacterial DNA were added.  
 PCR was performed (2 min 94°C, 30 cycles: 30 sec 94°C, 30 sec 52°C, 45 sec 52°C, 45 sec 52°C, 8 min 72°C)  
 The quality of PCR product was checked by agarose electrophoresis.

**Bacteria specific hybridization**  
 20 µl of the PCR product were mixed with 20 µl of the denaturation solution in the well of the tray  
 and incubated at room temperature for 5 min.  
 1 ml of prewarmed (50°C) hybridization buffer was added to the sample with a strip hybridized with  
 DNA sequence of each bacteria as well as a positive control was placed in the well of the tray.  
 The tray was incubated at 40°C for 30 min in a shaking water bath.  
 1 ml of transport media solution was added and incubated at 40°C for 30 min.  
 The strip was washed three with 1 ml of more solution for 1 min and 1 ml of conjugate solution  
 was added (room temperature for 20 min).  
 After washing 1 ml of substrate solution was added.  
 The occurrence of bacteria was evaluated visually by means of color change.  
 Two positive controls for amplification reaction and for conjugate were included in the test.



## Results and discussion

### Clinical characterization of the patient groups

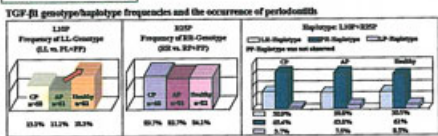
	Clinical periodontitis (CP)		Aggressive periodontitis (AP)		Healthy controls		p values vs. controls
	n=68	n=61	n=61	n=61	n=62	n=62	
Mean age (years)	43.2±5.6	42.2±3.3	44.4±2.7	44.4±2.7	44.4±2.7	44.4±2.7	0.1
Gender (M/F)	46/22	45	21/3	21/3	31/31	31/31	0.1
Smoking (N)	25	34	6	6	0	0	<0.001
Aggressive plaque index (AI)	41.8±25.3	31.8±20.3	43.3±21.4	43.3±21.4	<0.001	<0.001	0.1
Bleeding on probing (BOP)	70.3±34.6	70.2±32.2	40.2±19.9	40.2±19.9	<0.001	<0.001	0.1
Pocket depth (mm)	3.2±1.2	3.2±1.4	2.6±0.7	2.6±0.7	<0.001	<0.001	<0.001
Pocket depth >= 4 mm	6.7±1.6	7.2±1.6	1.2±0.4	1.2±0.4	<0.001	<0.001	<0.001
interdental loss (mm)	4.2±1.3	4.5±1.5	3.2±0.8	3.2±0.8	<0.001	<0.001	<0.001
Clinical attachment loss (mm)	7.4±1.9	8.4±1.8	3.3±0.8	3.3±0.8	<0.001	<0.001	<0.001
interdental loss (mm)							

No significant differences between the patient groups and the healthy control group could be proven investigating genetic, smoking status, and age. This exception was the age of the patients suffering from aggressive periodontitis because of the young age of onset of disease. As expected, both patient groups showed significantly more severe clinical symptoms compared to the control group.

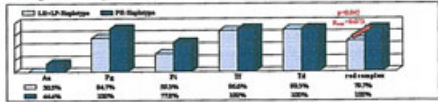
	CP	AP	Healthy controls	p values vs. controls
Aggregatibacter actinomycetemcomitans (AO)	30.6	43.3	32.3	0.1
Porphyromonas gingivalis (PG)	86.6	76.3	33.5	<0.001
Prevotella intermedia (PI)	61.8	41.7	32.9	<0.001
Tannerella forsythia (TF)	97.1	84.4	48.2	<0.001
Treponema denticola (TD)	98.5	82.3	44.7	<0.001
Fig, Tg, Tf (N)	62.4	71.3	33.5	<0.001

As expected, both patient groups showed distinct and nearly significant increase in the occurrence of periodontopathic bacteria. Interestingly, no significant difference in the subgingival colonization with Ag could be shown for patients suffering from CP.

### Genetic evaluation



**TGF-β1 genotype/haplotype frequencies and the occurrence of periodontitis**



**Hardy-Weinberg criteria were fulfilled for both TGF-β1 SNPs L18F and R23F.**  
 No significant associations of the genetic background of TGF-β1 including genotype and haplotype analysis and the occurrence of either chronic or aggressive periodontitis could be proven. A trend for a lower occurrence of the L18F genotype in patients suffering from aggressive periodontitis could be demonstrated (p=0.07). Furthermore, a tendency towards a lower occurrence of L18F haplotypes among patients with chronic periodontitis could be described (p=0.04). Among patients with chronic periodontitis bacteria of the red complex (Pg, Tg, Tf) occurred less frequently in carriers of the R23F haplotype (p=0.042). After Yates correction the association between red complex and R23F was not significant (p=0.487). However, in binary logistic regression analyses the SNPs L18F and R23F and haplotypes could not be proven as independent risk factors for bacterial colonization considering age, gender, smoking and AIT as cofactors.