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Is the genetic variability in genes of the IL-1 cluster associated with the subgingival occurrence of periodontopathogens?

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Regensburg, Germany

Introduction

Interleukin 1 cluster
IL-1a and 1b, its receptor (IL-1R) an receptor agonist (IL1-RA) are important factors in mediating pathogen dependend regulation of the immune system.
Functionally important genetic variants of the genes concerning to this IL-1 cluster are described and have been implicated in the pathogenesis of periodontitis.
However, results are conflicting.

Interleukin 1 and periodontitis
Periodontitis is characterized as a chronic inflammatory disease induced by periodontopathogens. Mechanisms modulating individual host's immune response play a crucial role in disease progression.
Genes of the IL-1 cluster influence many inflammatory cells (natural killer cells, macrophages, TH cells, B cells) which are of great importance in periodontal disease.
Functional important polymorphisms are described for IL-1a (rs1800587), IL-1b (rs16944, rs1143634), IL-1R (rs2234650), IL-1RA (rs315952).
A periodontitis associated composite genotype comprised of the rare genotypes of rs1800587 (IL-1a) and rs1143634 (IL-1b) has been described (Kornamn et al., 1997).

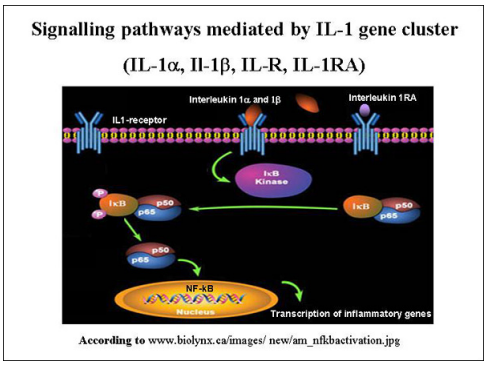


Fig. 1

Aim of the study

In the present study possible associations were investigated between the genetic variants of genes in IL-1 cluster and chronic/aggressive periodontitis and ist clinical features, including smoking status, plaque (API) and bleeding indexex (BOP), pocket depth (PD), clinical attachment loss (CAL) and subgingival bacterial colonization.

Material and Methods

Inclusion criteria of probands

Generalized aggressive periodontitis (AP, n=86):
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=73):
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=89):
probing depth ≤ 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 investigations

DNA-isolation from EDTA-blood
Preparation of genomic DNA from human venous EDTA-blood was carried out using the blood extraction kit (Quiagen).
200µl EDTA-blood and 20 µl protease were mixed in a 1,5 ml tube.
After adding of 200 µl denaturation buffer AL and pulse-vortexing for 15 sec the samples were incubated at 56°C for 10 min.
200 µl of ethanol was added to the samples, vortexed and the samples were applied to a QIAamp Spin Column were the DNA is bound.
After two washing steps (buffer AW1 and AW2) the DNA bound to the column is dried by centrifugation.
200 µl 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.

Genotype specific PCR of IL-1 gene cluster
The detection of genotypes 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 alleles prepipetted and lyophilized in thin-walled plastic 96-well PCR trays.
For every PCR 10µl of a Mastermix containing 1U Taq-Polymerase (Invitek), 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

Subgingival sampling
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 µl ATL-buffer and 20 µl proteinase K and incubated at 70°C for 10 min.
200 µl buffer Al 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 µl 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, biotynilated primer, DNA for positive control), 2U Taq-polymerase (Eppendorf), and 5 µl of isolated bacgterial 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 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 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.

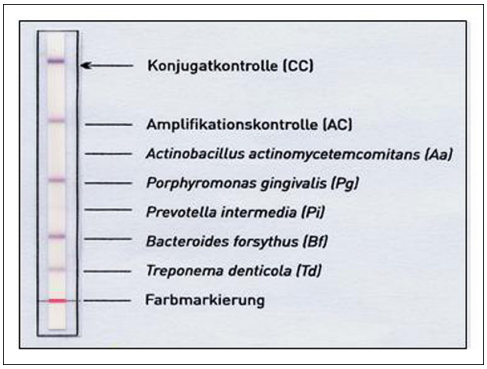


Fig. 2: Identification of subgingival bacteria (HAIN-diagnostics)

Results

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. 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.

	Chronic periodontitis (CP)	**Aggressive periodontitis (AP)**	**healthy controls**	**p values vs. controls**	
	n=73	n=86	n=89	CP	AP
Mean age (years)	49.1±9.4	40.4±9.8	46.2±10.8	n.s.	< 0.001
Gender (% female)	63.0	64.0	53.3	n.s.	n.s.
Smoking (%)	23.6	34.9	21.3	n.s.	n.s.
Approximal plaque index (%)	62.0±25.6	53.3±28.7	47.2±21.4	< 0.001	n.s.
Bleeding on probing (%)	70.6±24.7	78.7±23.2	45.2±23.9	< 0.001	< 0.001
Pocket depth (mm)	5.3±1.3	5.7±1.4	2.6±0.7	< 0.001	< 0.001
Clinical attachment loss in general (MM)	6.0±1.5	6.5±1.5	3.0±0.8	< 0.001	< 0.001
Teeth with CAL 4-6mm (%)	45.8	39.5	3.4	< 0.001	< 0.001
Teeth with CAL > 6mm (%)	44.4	57.0	1.1	< 0.001	< 0.001
Early tooth loss due to periodontitis among relatives	40.9	57.0	9.1	< 0.001	< 0.001

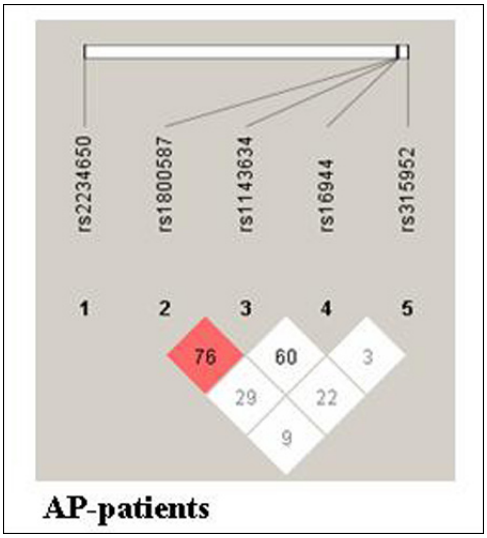
Tab. 1: Clinical and demographic characterization of the patient groups

	Chronic periodontitis (CP)	**Aggressive periodontitis (AP)**	**healthy controls**	**p values vs. controls**	
	n=73	n=86	n=89	CP	AP
Aggregatibacter actinomycetemcomitans (%)	34.2	40.7	18.0	n.s.	0.001
Porphyromonas gingivalis (%)	87.7	76.7	22.5	< 0.001	< 0.001
Prevotella intermedia (%)	61.6	61.6	31.5	< 0.001	< 0.001
Tannerella forsythia (%)	97.3	86.0	68.5	< 0.001	0.005
Treponema denticola (%)	98.6	86.0	62.9	< 0.001	0.002
Pg, Td, Tf (%)	83.6	69.8	22.5	< 0.001	< 0.001

Tab. 2: Microbiological characterization of the patient groups

Genetic evaluation

Between rs1800587 and rs1143634 a strong linkage disequilibrium could be shown for patients suffering from periodontitis as well as periodontitisfree controls (LOD > 10).



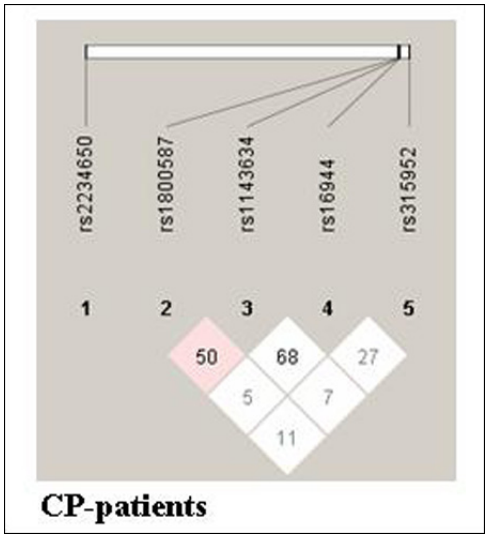
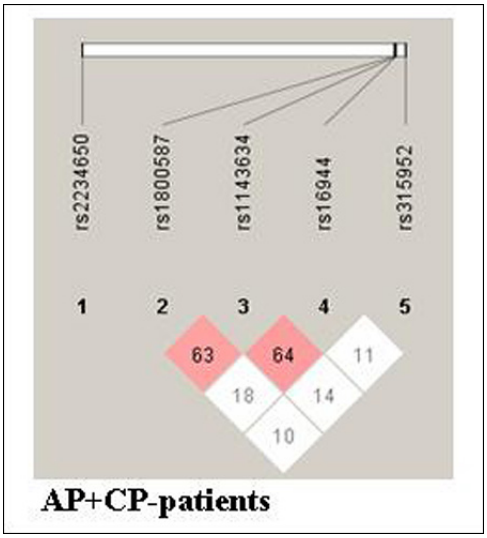


Fig. 3a-b: Genetic evaluation: Haplotype block structure determined using Haploview 4.2



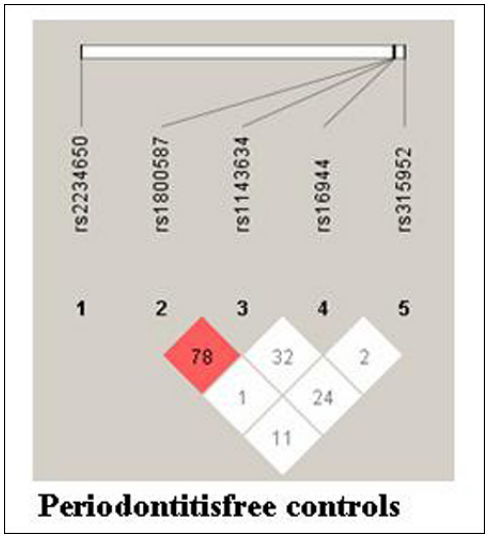


Fig. 3c-d: Genetic evaluation: Haplotype block structure determined using Haploview 4.2

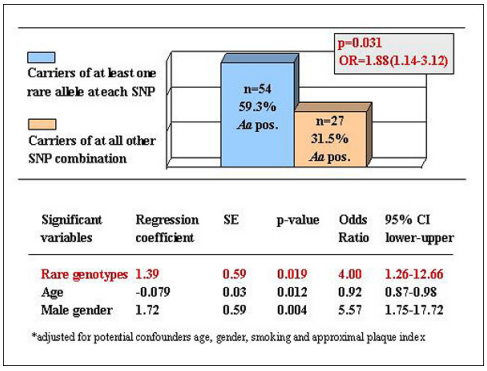


Fig. 4: Genotype dependent evaluation: Composite genotype (IL-1a, rs1800587 + IL-1b, rs1143634) and A.actinomycemtcomitans in AP-patients

Genotype dependent evaluation

	AP (n=86)	CP (n=73)	Periodontitisfree controls (n=89)
IL-1α: rs1800587			
TT (%)	7.1	6.9	7.9
CT + TT (%)	45.9	50.0	50.6
IL-1β: rs16944			

TT (%)	15.1	8.2	14.6
CT + TT (%)	57.0	56.2	59.6
IL-1β: rs1143634			
CC (%)	62.2	61.1	56.8
CT + TT (%)	37.8	38.9	43.2
IL-1R: rs2234650			
CC (%)	48.8	42.5	46.1
CT + TT (%)	51.2	57.5	53.9
IL-1RA: rs315952			
TT (%)	45.3	46.6	40.9
CT + CC (%)	54.7	53.4	59.1
Composite genotype I: IL-1α: rs1800587 + IL-1β: rs1143634			
Carrier of all other SNP combination (%)	33.3	31	40.9
Carrier of at least one rare allele at each SNP (%)	66.7	69	59.1

Tab. 3: Genotype dependent evaluation: Genotype and allele distribution of polymorphsims in IL-1 gene cluster in dependence on the occurrence of AP and CP

Conclusions

Despite the genetic background of IL-1 gene cluster could be shown to be associated with subgingival colonization of A. actinomycetemcomitans there is no evidence that it is an independent risk modulator for periodontitis.

This Poster was submitted by Dr. Susanne Schulz.

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Poster Faksimile:

22. Jahrestagung der Deutschen Gesellschaft für Humangenetik in Regensburg vom 16. - 18.03.2010

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[1] University School of Dental Medicine, Department of Operative Dentistry and Periodontology, Martin-Luther-University Halle-Wittenberg
[2] Institute of Human Genetics and Medical Biology, Martin-Luther-University, Halle-Wittenberg
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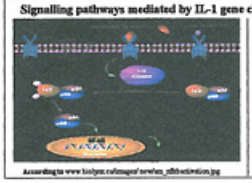
Introduction

Signalling pathways mediated by IL-1 gene cluster (IL-1α, Il-1β, IL-R, IL-1RA)



According to www.biotype.ru/images/newsen_nfkbactivation.jpg

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However, results are conflicting.

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A periodontitis associated composite genotype comprised of the rare genotypes of rs1800587 (IA–1α) and rs1143634 (IL-1β) has been described (Kornman et al., 1997).

Aim of the study:
In the present study possible associations were investigated between the genetic variants of genes in IL-1 cluster and chronic/aggressive periodontitis and bit clinical features, including *smoking status, plaque (API) and bleeding indexes (BOP), pocket depth (PD), clinical attachment loss (CAL) and subgingival bacterial colonization.*

Material and Methods

Inclusion criteria of probands

Generalized aggressive periodontitis (AP): n=86
- clinical manifestation before 35th year of life
- attachment loss at at least 30% of the teeth with a maximum pocket depth of 4 mm
- > 3 affected teeth had to be on first molars or incisors
- severity of attachment loss was inconsistent to the amount of mineralized plaque
- more vertical than horizontal appraisal bone loss was visible in Rö-radiographs

Generalized chronic periodontitis (CP): n=75
- attachment loss at at least 30% of the teeth with a maximum pocket depth of 4 mm
- The amount of the attachment loss was consistent with the presence of mineralized plaque
- More horizontal than vertical appraisal bone loss was visible in Rö-radiographs

Periodontitisfree controls: n=89
- probing depth ≤ 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

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For every PCR a fragment of 448bp of the human CRP gene was examplified as a positive control.
The PCRs were performed using sequence specific primers for detection of possible allele propagated and lyophilized in thin-walled plastic 96-well PCR trays.
For every PCR 10µl of a Mastermix containing TU Taq-Polymerase (Invitek), 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 66°C, 20 cycles: 15 sec 94°C, 30 sec 61°C, 30 sec 72°C)
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Paper points for collection of subgingival samples were used to bind periodontopathogens of the deepest pocket of each quadrant.

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Preparation of bacterial DNA was carried out using the QIAamp DNA Mini Kit (Qiagen).
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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 (Sigma-Aldrich), and 5 µl 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 51°C, 40 sec 70°C; 8 min 70°C)
The quality of PCR product was checked by agarose gel 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 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).
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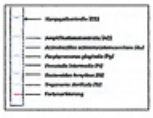
The occurrence of bacteria was evaluated visually by means of colored bands.
Two positive controls for amplification reaction and for conjugate were included in the test.



Results and discussion

Clinical characterization of the patient groups

Clinical and demographic characteristics

	Chronic periodontitis (CP) n = 75	Aggressive periodontitis (AP) n=86	healthy controls n=89	p values vs. controls CP	AP
Mean age (years)	49.1±9.4	40.4±9.8	46.2±10.8	n.s.	<0.001
Gender (% female)	63.0	64.0	53.3	n.s.	n.s.
Smoking (%)	33.6	34.9	23.3	n.s.	n.s.
Approximal plaque index (%)	62.0±25.4	53.3±26.7	47.2±21.4	<0.001	n.s.
Bleeding on probing (%)	70.4±24.7	78.7±23.2	45.2±23.9	<0.001	<0.001
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Teeth with CAL 4-6mm (%)	43.8	39.5	3.4	<0.001	<0.001
Teeth with CAL >6mm (%)	44.4	37.0	1.1	<0.001	<0.001
Early tooth loss due to periodontitis among relatives	46.9	37.0	9.1	<0.001	<0.001

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.

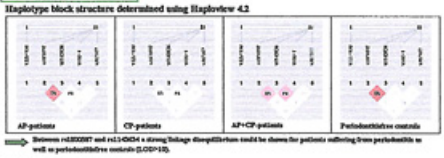
Microbiological assessment

Aggregatibacter actinomycetemcomitans (%)	34.2	48.7	18.0	n.s.	0.001
Porphyromonas gingivalis (%)	87.7	74.7	32.5	<0.001	<0.001
Prevotella intermedia (%)	61.6	61.6	31.5	<0.001	<0.001
Tannerella forsythia (%)	97.3	96.0	68.5	<0.001	0.005
Treponema denticola (%)	98.6	86.0	62.9	<0.001	0.002
Pg, Tf, Td (%)	83.6	69.8	32.5	<0.001	<0.001

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 *An* could be shown for patients suffering from CP.

Genetic evaluation

Haplotype block structure determined using Haploview 4.2

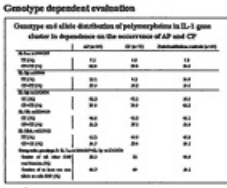


AP-patients | CP-patients | AP+CP-patients | Periodontitisfree controls

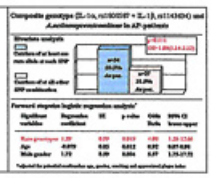
Between rs1800587 and rs1143634 a strong linkage disequilibrium could be shown for patients suffering from periodontitis as well as periodontitisfree controls (LOD>10).

Genotype dependent evaluation

Genotype and allele distribution of polymorphisms in IL-1 gene cluster in dependence on the occurrence of AP and CP



Composite genotype (IL-1α, rs1800587 + IL-1β, rs1143634) and *A.actinomycetemcomitans* in AP-patients



Forward stepwise logistic regression analysis*

Significant variables	Regression coefficient	SE	p-value	Odds Ratio	95% CI lower/upper
Non-genotype	1.37	0.57	0.015	3.95	1.28/12.16
Age	-0.059	0.02	0.012	0.92	0.87/0.96
Male gender	1.73	0.59	0.004	5.67	1.79/17.73

*adjusted for potential confounders age, gender, smoking and aggressive plaque index

Despite the genetic background of IL-1 gene cluster could be shown to be associated with subgingival colonization of *A. actinomycetemcomitans* there is no evidence that it is an independent risk modulator for periodontitis.