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The role of genetic polymorphisms and the subgingival occurrence of periodontopathogens

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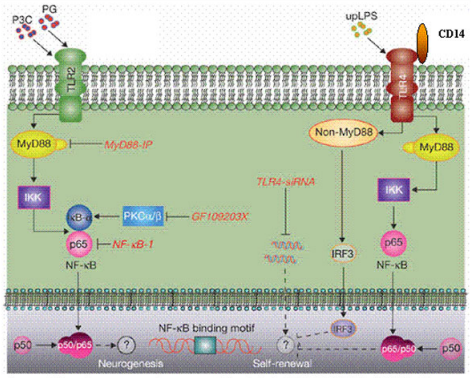
Introduction

Periodontitis is characterized as a chronic inflammatory disease induced by periodontotpathogens. Molecular patterns of periodontopahtogens induce immune response amongst others via CD14 and re-ceptors of the Toll-like receptor (TLR) family, including TLR2 and TLR4 resulting in activation of NF-kB.

Functional important polymorphisms are described for those genes:
CD14: c.-159C>T TT-genotype enhances transcription rate Hubacek et al., 1999
TLR2: Arg677Trp mutant allele abolishes NF-kB activation Bouchud et al., 2003
Arg753Gln important for TLR2 signalling Gautam et al., 2006
TLR4: Asp299Gly associated with diminished responsiveness to LPS Rhallabandi et al., 2006
Thr399Ile associated with diminished responsiveness to LPS Rhallabandi et al., 2006
NF-kB: -94ins/delATTG associated with transcriptional expression Karban et al., 2004

Aim of the study

In the present study possible associations were investigated between the individual genetic background based on SNPs in CD14, TLR2, TLR4 and NF-kB and the subgingival occurrence of periodontopthogens, including Aggregatibacter actinomycetemcomitans (A.a.), Porphyromonas gingivalis (P.g.), Prevotella intermedia (P.i.), Tannerella forsythensis (T.f.), Treponema denticola (T.d).



According to www.nature.com/fig_tab/ncb0907-1031_F1.html

CD14 and receptors of the Toll-like receptor (TLR) family, including TLR2 and TLR4 play a fundamental role in pathogen recognition and activation of innate immunity.

The receptors recognize pathogen-associated molecular patterns and mediate the production of cytokines necessary for the immune response via the activation of transcription factor NF-κB.

Because NF-κB controls many genes involved in inflammation it is found to be chronically active in many inflammatory diseases, including periodontitis.

Fig. 1: Signalling pathways induced by CD14 and Toll like receptors 2 and 4

Material and Methods

Inclusion criteria of probands

Generalized aggressive periodontitis (AP) n=78:
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=63:
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=81:
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.
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 actinomycetemcomitan, 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.

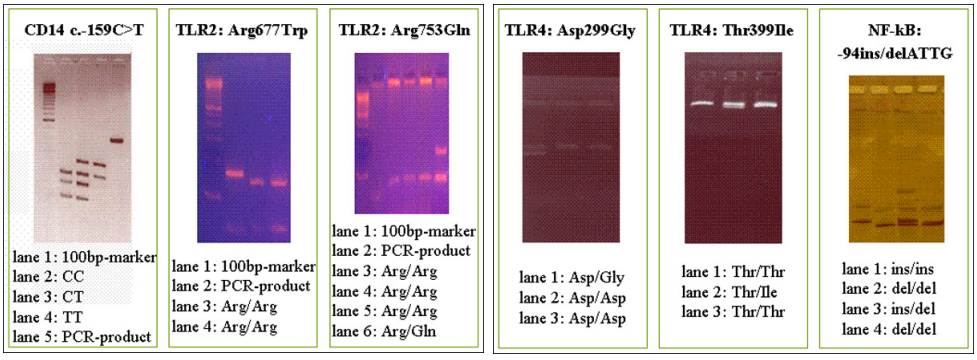


Fig. 2-3: Genotyping by means of fragment length analyses

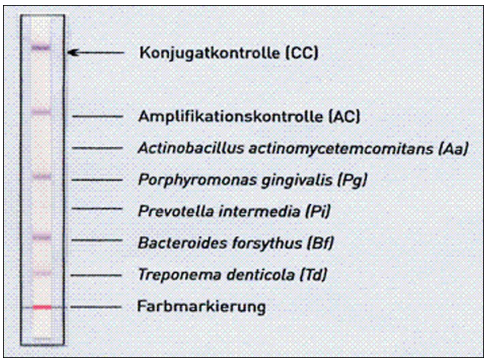


Fig. 4: Analysis of periodontopathic bacteria in subgingival pockets using micro-Ident® test of HAIN-Diagnostik

Results

Clinical characterization of the patient groups:

Clinical and demographic characterization

No significant differences between the patient groups and the healthy control group could be proven investigating age, gender and smoking status. As expected, both patient groups showed significant more severe clinical symptoms compared to the control group.

	Chronic periodontitis (CP)	**Aggressive periodontitis (AP)**	**healthy controls**	**p values vs. controls**	
	n=63	n=78	n=81	CP	AP
Mean age (years)	48.6 ± 9.6	40.6 ± 9.9	46.6 ± 10.9	n.s.	< 0.001
Gender (%female)	65.1	61.5	51.9	n.s.	n.s.
Smoking (%)	28.8	35.9	30.5	n.s.	n.s.
Approximal plaque index (%)	60.5 ± 25.6	52.3 ± 28.5	47.5 ± 21.5	< 0.001	0.005
Bleeding on probing (%)	69.5 ± 24.9	78.1 ± 22.7	45.7 ± 23.7	< 0.001	< 0.001
Pocket depth (mm)	5.2 ± 1.1	5.7 ± 1.4	2.6 ± 0.8	< 0.001	< 0.001
Pocket depth on microbial test site (mm)	6.8 ± 1.5	7.5 ± 1.6	3.1 ± 0.4	< 0.001	< 0.001
Clinical attachment loss in general (mm)	5.9 ± 1.4	6.5 ± 1.5	3.0 ± 0.8	< 0.001	< 0.001
Clinical attachment loss on microbial test site (mm)	7.5 ± 1.8	8.4 ± 1.8	3.3 ± 0.5	< 0.001	< 0.001

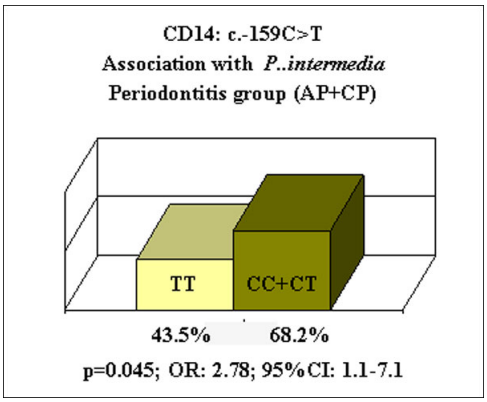
Tab. 1: Clinical and demographic characterization of the probands

Microbiological assessment

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.

Aggregatibacter actinomycetemcomitans (%)	31.7	41.0	19.8	n.s.	0.001
Porphyromonas gingivalis (%)	87.3	78.2	23.5	< 0.001	< 0.001
Prevotella intermedia (%)	61.9	64.1	33.3	< 0.001	< 0.001
Tannerella forsythia (%)	96.8	85.9	69.1	< 0.001	< 0.001
Treponema denticola (%)	98.4	87.2	64.2	< 0.001	0.002
Pg, Td, Tf (%)	82.5	71.8	23.5	< 0.001	< 0.001

Tab. 2: Microbiological assessment



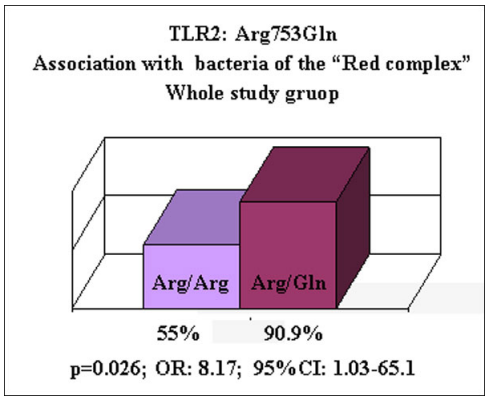


Fig. 5-6: Genetic evaluation: Bivariate associations between genotype an the subgingival occurrence of periodontopathogens

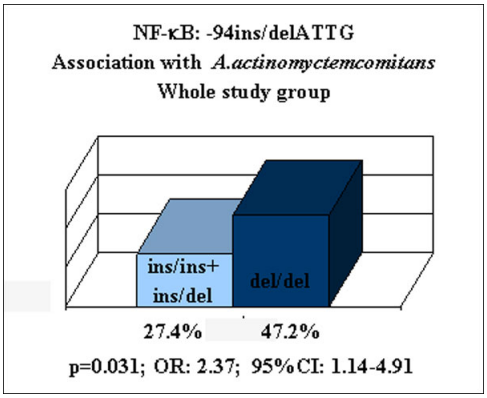


Fig. 7: Genetic evaluation: Bivariate associations between genotype an the subgingival occurrence of periodontopathogens

CD14: c.-159C>T;

Significant variables	Regression coefficient	SE	p-value	Odds Ratio	95% CI
Male gender	1.1	0.42	0.010	2.99	1.3-6.9
Genotype CC+CT	1.18	0.49	**0.017**	3.23	1.23-8.33

*adjusted for age, gender, smoking, clinical attachment loss at microbiological test site

NF-κB: -94ins/delATTG

Significant variables	Regression coefficient	SE	p-value	Odds Ratio	95% CI
PDbacteria	0.146	0.07	0.026	1.16	1.01-1.32
Genotype del/del	0.842	0.39	**0.033**	2.32	1.07-5.03

*adjusted for age, gender, smoking, probing depth at microbiological test site (PDbacteria)

Tab. 3: Genetic evaluation: Multivariate risk analyses: forward stepwise binary logistic regression analyses

Conclusions

SNPs in candidate genes responsible for the bacterial recognition and elimination could be shown to be associated with the subgingival occurrence of periodontopathogens in bivariate analyses.
In multivariate analyses considering further cofounders of periodontitis the CT+CC genotype of CD14 SNP and the del/del genotype of NF-kB SNP could be proven as independent risk factor for subgingival occurrence of P.intermedia and A.actinomycetemcomitans, respectively.
The analysis of disease related genetic pattern may help to identify early persons at high periodontal risk and improve success of the periodontal therapy.

This Poster was submitted by Dr. Susanne Schulz.

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Poster Faksimile:

Poster P003, 20. Jahrestagung der Deutschen Gesellschaft für Humangenetik in Aachen vom 01. - 03.04.2009

The role of genetic polymorphisms and the subgingival occurrence of periodontopathogens

S Schulz [1], L Hierse [1], N Zissler [1], W Altermann [2], J Klapproth [1], U Zimmermann [1], C Gläser [3], HG Schaller [1], S Reichert [1]

[1] University School of Dental Medicine, Department of Operative Dentistry and Periodontology, Martin-Luther-University Halle-Wittenberg
[2] Interbranch HLA Laboratory / Department GHATT, Martin-Luther-University Halle-Wittenberg
[3] Institute of Human Genetics and Medical Biology, Martin-Luther-University, Halle-Wittenberg

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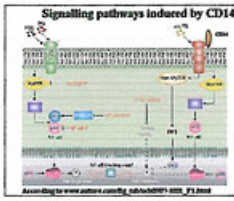
Signalling pathways induced by CD14 and Toll like receptors 2 and 4



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Because NF-κB controls many genes involved in inflammation it is found to be chronically active in many inflammatory diseases, including periodontitis.

Material and Methods

Inclusion criteria of probands

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n=70
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): attachment loss in at least 30% of the teeth with a minimum pocket depth of 4 mm
n=63 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

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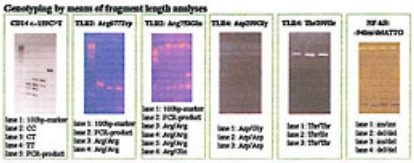
Genotyping by means of fragment length analyses



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Biotinylin provided in the micro-IdentB test (containing buffer, biotinylated primer, DNA for positive control), 27 Taq-polymerase (Eppendorf), and 5 µl of isolated bacterial DNA were mixed.
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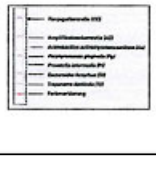

Results and discussion

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Smoking (%)	20.8	23.9	30.5	n.s.	n.s.
Approximal plaque index (%)	69.7±25.4	52.3±20.5	47.7±21.3	<0.001	0.005
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⇒ **No significant differences between the patient groups and the healthy control group could be proven investigating age, gender and smoking status. As expected, both patient groups showed significant more severe clinical symptoms compared to the control group.**

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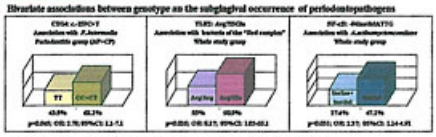
⇒ **As expected, both patient groups showed distinct and mostly significant increase in the occurrence of periodontopathic bacteria. Interestingly, no significant difference in the subgingival colonization with *Aa* could be shown for patients suffering from CP.**

Genetic evaluation

Bivariate associations between genotype an the subgingival occurrence of periodontopathogens



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Genotype CC+CT	1.16	0.49	0.017	3.13	1.23-8.33	Genotype del/del	0.942	0.39	0.035	2.32	1.07-5.03

*adjusted for age, gender, smoking, clinical attachment loss at microbiological test site

⇒ **SNPs in candidate genes responsible for the bacterial recognition and elimination could be shown to be associated with the subgingival occurrence of periodontopathogen in bivariate analyses.**
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