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Variants of the human *NR1I2* (*PXR*) locus in chronic periodontitis

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Background and Objective: Recent studies on genetic variants of the pregnane X receptor (PXR) revealed associations with the mucosal immune response. This study aimed to investigate the potential association of functional polymorphisms of the *NR1I2* (*PXR*)-encoding gene (rs12721602, rs3814055, rs1523128, rs1523127, rs45610735, rs6785049, rs2276707 and rs3814057) with chronic periodontitis.

Material and Methods: At total of 402 periodontitis patients and 793 healthy individuals were genotyped using PCR and melting-curve analysis.

Results: Frequency distribution of genotypes for the eight single nucleotide polymorphisms showed no significant difference between patients with periodontitis and controls. Among the eight tested polymorphisms, two blocks were defined showing complete or almost complete linkage disequilibrium (linkage disequilibrium block 1: rs3814055 and rs1523127; and linkage disequilibrium block 2: rs6785049, rs2276707 and rs3814057). For one haplotype (GTGAG) composed of rs12721602, rs3814055, rs1523128, rs12721607 and rs6785049, a significant association with periodontitis was found [*p*-value after permutation with 100,000 iterations ($p_{permut.}$) = 0.011, odds ratio = 0.46, 95% confidence interval: 0.25–0.84) following adjustment for age, gender and smoking.

Conclusion: A rare haplotype of the *NR1I2* (*PXR*) locus was associated with the individual susceptibility for chronic periodontitis in a German cohort. As a result of the borderline significance and the small effect size the present results need further confirmation.

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Pregnane X receptor (PXR), also known as steroid and xenobiotic receptor (SXR), belongs to a large family of nuclear receptors that act as xenobiotic sensors. PXR is a ligand-activated transcription factor that has regulatory effects on a broad spectrum of physiological processes which allow drug clearance via the induction of genes involved in drug and xenobiotic metabolism (1). Apart from its role in the metabolism of xenobiotics, PXR has been shown to be complicit in the regulation of inflammatory processes. Recently, two groups proposed that cross-talk exists between PXR and nuclear factor-κB (NF-κB), providing a potential biologic link between xenobiotic metabolism and inflammation (2,3). Activated human PXR inhibits NF-κB-mediated reporter activity and the expression of NF-κB target genes (4). Knockout mice deficient in PXR showed a marked increase of NF-κB expression in various tissues (5). Inversely, a stronger expression of NF-κB inhibited PXR activity and the expression of NF-κB target genes.

Based on an extensive resequencing analysis of NR1/2, Zhang *et al*. identified a total of 38 polymorphisms in the human *PXR* gene, among which different variants have been associated with altered PXR function (5,6). Although not reported as susceptibility

loci in recent genome-wide searches, several studies have found that some of the *PXR* gene variants are associated with the susceptibility to inflammatory bowel disease (7,8). In addition, variants at the *PXR* locus seem to have impact on the clinical course of primary sclerosing cholangitis (9). However, the biologic effects of these single nucleotide polymorphisms (SNPs) on the function of PXR remain to be elucidated.

Periodontitis is a chronic inflammatory disease of the oral cavity (10) that has a strong genetic background (11). The rate of concordance for chronic periodontitis among monozygotic twins has been previously reported to be as high as 50%, clearly showing the contribution of genetic and environmental components to the risk of developing periodontitis (11). The genetic impact on susceptibility to and/or severity of periodontitis in an individual seems to be mediated by a dysregulated immune response against the bacterial colonization of the periodontal pocket (12). Various susceptibility genes have been identified so far, which are linked to the innate immune response. These include the genes coding for interleukin-1, interleukin-6, tumor necrosis factor-α, CD14 receptor and toll-like receptors (13). Also, NF-κB plays seems to play a central role in the mediation and regulation of the periodontal immune response (14).

Recent studies have speculated that inflammatory bowel disease and periodontitis share some etiological factors (15). Chemical stimulation using trinitrobenzene sulfonic acid led to the coincidental development of intestinal mucosal inflammation and inflammatory alveolar bone loss in mice (16). Hence, variants of genes that are involved in the regulation of the NF-κB pathway and that are associated with inflammatory bowel disease are excellent candidates to elucidate the genetic background of chronic periodontitis.

We hypothesized that polymorphisms altering *PXR* expression or function may affect the disease susceptibility or course in chronic periodontitis.

Material and methods

Patient population

Selection of study participants was performed on the basis of a standardized periodontal examination procedure, as described previously (17). The diagnosis of periodontitis was made on the basis of the following clinical and radiographic criteria: (i) a total of at least 15 teeth *in situ*; (ii) eight or more teeth with a diagnosis of periodontitis clinically defined as a probing pocket depth of ≥ 5 mm and/or attachment loss of ≥ 2 mm at least at one location and/or a furcation involvement of ≥Class II. For all individuals with periodontitis an additional radiographic examination was performed using orthopantomographs. Radiographic bone level was determined as the distance between the alveolar crest and the cemento–enamel junction. If this distance was ≥ 3 mm a diagnosis of periodontitis was made for the affected tooth. The absence of periodontal disease in control individuals was determined according to the following criteria: (i) a minimum of 22 teeth *in situ*; (ii) one site or fewer with a probing pocket depth of ≥ 3 mm or any attachment loss; and (iii) lack of any kind of furcation involvement at any tooth. Control individuals did not have a history of periodontitis and had not received periodontal treatment in the past. For ethical reasons radiographic examination of the periodontal status of healthy subjects was performed only if an orthopantomograph was indicated.

All individuals enrolled into both study groups were adult Caucasians of German descent from one geographic region. Information regarding smoking habit was available for 368 of the patients with periodontitis and for 781 of the controls. Individuals were classified as smokers if they had a self-reported history of smoking of at least 10 cigarettes per day for at least 1 year.

Pregnant women and individuals with severe medical disorders, including diabetes mellitus, immunological disorders and an increased risk for bacterial endocarditis, were excluded from the study. The study conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the local Ethics Committee. All participants provided written informed consent before enrolment into the study.

Blood samples and DNA isolation

Peripheral venous blood samples of 9 mL were drawn from each individual by standard venepuncture. Each blood sample was collected in sterile tubes containing K_3EDTA solution. DNA was isolated partly using the QIAamp® DNA Blood Midi Kit (Qiagen, Hilden, Germany) and partly using the salting-out procedure (18).

Genotyping of the polymorphisms within the *PXR* gene

The eight *PXR*/*NR1I2* gene variants rs12721602 (−25564; G/A), rs3814055 (−25385; C/T), rs1523128 (−24756; G/A), rs1523127 (−24381; A/C), rs456107351 (+106; G/A), rs6785049 (+7635; A/G), rs2276707 (+8055; C/T) and rs3814057 (+11156; A/C), located along the gene from the promotor region to the 3′ untranslated region, were genotyped (the numbers in parenthesis correspond to the distance from the transcription start point, and the letters indicate the base exchange at this position for the positive strand). For genotyping, the PCR and melting curve analysis were used with a pair of fluorescence resonance energy transfer (FRET) probes in a LightCycler 480 Instrument (Roche Diagnostics, Mannheim, Germany), as described by Glas *et al.* (7). All sequences of primers and FRET probes and primer-annealing temperatures used for genotyping and for sequence analysis can be provided upon request.

Statistical analysis

From *post-hoc* analysis using a dominant model, the power of the present study sample was estimated to be 0.74 for a risk-allele frequency of 0.1 and 0.29 for a risk-allele frequency of 0.05 using the Gentic Power Calculator (19). The significance level for power analysis was set at $p < 0.05$. Coverage of genetic variance was determined using the method described by Jorgenson *et al.*

(20) and revealed that 35% of common SNPs (minor allele frequency ≥ 10%) had an $r^2 > 0.8$ with at least one of the genotyped SNPs in the region of chromosome 3 that has been tested (position 120982500–121011000). Adherence to Hardy–Weinberg equilibrium was tested in the control group separately for each genetic marker using an Exact test and a type 1 error level of 0.05 (21). Significance of associations with single-locus genotypes were assessed using Fisher's exact test or the Pearson chi-square test with Yates' correction, where appropriate. Correction for multiple testing was performed using the Bonferroni procedure considering *p*-values of < 0.006 as significant. Subgroup analysis was performed according to gender and smoking. For comparison of quantitative variables, the Student's *t*-test was used. For determination of the mode of inheritance, Akaike's Information Criterion was used (22). Each SNP was considered under a co-dominant, dominant, recessive and overdominant effect model using SNPStats (http://bioinfo.iconcologia.net/index.php?module=Snpstats). For each polymorphism, the odds ratio, 95% confidence interval and *p*-value were calculated using the best fitting model. Linkage disequilibrium (LD) measures were estimated using the HAPLOVIEW 4.2 software (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview). For polymorphisms in strong LD, blocks were defined according to an algorithm taken from Gabriel *et al.* (23). The haplotype frequencies were determined with the expectation maximation algorithm (24). Analysis of associations between haplotypes and phenotypes was performed with SNPStats after adjustment for age, gender and smoking as confounding variables. The permutation test (100,000 iterations) was used to adjust the *p*-values for multiple testing.

Results

Study sample

A total of 402 patients from the Department of Periodontology, Ludwig-Maximilians University (Munich, Germany) were enrolled in the study. The mean (± standard deviation) age in the periodontitis group was 54.1 (± 11.1) years and the age range was 18–85 years. The male to female ratio (%) was 47.5:52.5. A total of 793 unrelated, ethnically matched, individuals without periodontitis comprised the healthy control group. Within the control group the mean (± standard deviation) age was 47.3 (± 9.2) years and the age range was 18–73 years (Table 1).

Frequency distribution of alleles and genotypes

For each SNP the genotype frequencies among control individuals were in agreement with the Hardy–Weinberg equilibrium. Depending on the polymorphism, 97.7–98.5% of study subjects were successfully genotyped herein. Minor allele and genotype frequencies of the eight variants of the *PXR* gene within both study groups are shown in Table 2.

Association analysis revealed nominal significant differences in the frequency distribution of genotypes for rs12721607 between periodontitis patients and control individuals. However, this association remained nonsignificant following correction for multiple testing. None of the other tested *NR1I2* (*PXR*) variants showed significant associations with periodontitis (Table 3). When performing subclass analysis according to gender and smoking status, significant differences again were found in the frequency distribution of genotypes for rs12721607 among nonsmoking individuals but these were nonsignificant after applying the Bonferroni procedure (data not shown).

LD and haplotype analysis

The LD pattern for the *NR1I2* (*PXR*) variants is depicted in Fig. 1. Among the eight tested polymorphisms, two blocks were defined showing complete or almost complete LD. In LD block 1, rs3814055 and rs1523127 are found, whereas rs6785049, rs2276707 and 3814057 are located in LD block 2. A five-site haplotype analysis excluding rs1523127, rs2276707 and rs3814057 revealed a frequency of ≥ 1% for a total of six haplotypes in both study groups (Table 4). One of these haplotypes (G-T-G-A-G), composed of rs12721602, rs3814055, rs1523128, rs12721607 and rs6785049, showed a significant association with periodontitis [$p = 0.011$, odds ratio = 0.46, 95% confidence interval: 0.25–0.84; *p*-value after permutation with 100,000 iterations ($p_{permut.}$) = 0.031].

Discussion

Herein we performed a case–control study using eight polymorphisms in the *NR1I2* (*PXR*) gene. Although showing inconsistent results in different populations, previous studies revealed significant associations between three of these genetic variants (rs3814055, rs1523127 and rs6785049) and inflammatory bowel disease (i.e. Crohn's disease and ulcerative colitis) (7,8).

In the present study none of the *NR1I2* (*PXR*) gene variants was associated with susceptibility to chronic periodontitis, neither when considering the entire study population nor following stratification according to gender and smoking. However, haplotype analysis revealed that one haplotype (G-T-G-A-G), composed of the polymorphisms rs12721602, rs3814055, rs1523128, rs12721607 and rs6785049, is significantly associated with susceptibility to periodontal disease, albeit showing only borderline significance. Hence, the observed association remains to be confirmed in independent samples in order to exclude false-positive results. The observed association of the G-T-G-A-G haplotype seems to be caused primarily by the effect of rs1272607, which showed a nominal significant association with periodontitis without correction for multiple testing.

Different reasons may be given to explain the weak association between the *NR1I2* (*PXR*) gene and periodontitis. One might comprise the small effect size of the tested variants. As the haplotype in question is present at a very low frequency it seems to have only a minor impact on the overall disease risk. Interestingly, this

Table 1. Demographic data of the two study groups (periodontitis patients and healthy controls)

	Healthy controls (n = 793)	Periodontitis patients (n = 402)	p-value
Age at diagnosis (years)	47.3 (18–73)	54.1 (18–85)	< 0.001
Sex			
Male	531 (67.4)	178 (47.5)	
Female	257 (32.6)	197 (52.5)	< 0.001
Smoking			
Smokers	97 (12.4)	116 (31.5)	
Nonsmokers	684 (87.6)	252 (68.5)	< 0.001
Number of teeth	NA	25.3 ± 4.9	

Results are given as mean (range), n (%) or mean ± standard deviation.
NA, not available.

with inflammation may be primarily caused by differences in the allele frequencies in the healthy controls tested in these studies. The minor allele frequency in the healthy control group in the Irish sample was 43.3%, compared with 38.2% in the Spanish cohort and 37.3% in the present study. Hence, different explanations can be given, for example, genetic heterogeneity and cryptic population stratification, for the the divergence between the various populations.

Taken together, the present results in periodontitis patients, along with the

Table 2. Frequency distribution of genotypes for the eight single nucleotide polymorphisms (SNPs) at the *NR1I2* (*PXR*) locus

	No. (%) of periodontitis genotypes				No. (%) of control genotypes				
SNP (alleles)	*N* 11	*N* 12	*N* 22	MAF	*N* 11	*N* 12	*N* 22	MAF	HWE
rs12721602 (G/A)	373 (94.7)	21 (5.3)	0 (0.0)	2.7	745 (95.9)	31 (4.0)	1 (0.1)	2.1	0.29
rs3814055 (C/T)	133 (33.8)	205 (52.2)	55 (14.0)	40.1	302 (39.0)	369 (47.6)	104 (13.4)	37.3	0.64
rs1523128 (G/A)	393 (99.0)	4 (1.0)	0 (0.0)	0.5	769 (98.7)	10 (1.3)	0 (0.0)	0.6	1.00
rs1523127 (A/C)	129 (32.6)	210 (53.0)	57 (14.4)	40.9	751 (96.7)	26 (3.3)	0 (0.0)	38.6	0.94
rs12721607 (G/A)	371 (93.5)	26 (6.5)	0 (0.0)	3.3	751 (96.7)	26 (3.3)	0 (0.0)	1.7	1.00
rs6785049 (A/G)	158 (39.7)	184 (46.2)	56 (14.1)	37.2	301 (38.6)	376 (48.3)	102 (13.1)	37.2	0.40
rs2276707 (C/T)	274 (69.2)	111 (28.0)	11 (2.8)	16.8	515 (66.3)	238 (30.6)	24 (3.1)	18.4	0.63
rs3814057 (A/C)	273 (69.3)	111 (28.2)	10 (2.5)	16.6	513 (66.1)	239 (30.8)	24 (3.1)	18.5	0.63

N, number of individuals; 1, major allele; 2, minor allele; MAF, minor allele frequency; HWE, analysis of Hardy–Weinberg equilibrium.

Table 3. Analysis of the frequency distribution of genotypes of the eight single nucleotide polymorphisms (SNPs) at the *NR1I2* (*PXR*) locus between patients with periodontitis and healthy controls

SNP	OR(cr)	95% CI	OR(adj)	95% CI	p-value	Model
rs12721602	0.74	0.42–1.30	0.67	0.36–1.27	0.3	Overdominant
rs3814055	0.80	0.62–1.03	0.85	0.63–1.14	0.09	Dominant
rs1523128	1.28	0.40–4.10	1.06	0.29–3.90	0.68	Log-additive
rs1523127	0.79	0.62–1.01	0.76	0.57–1.00	0.06	Overdominant
rs12721607	0.45	0.28–0.86	0.52	0.28–0.99	0.007	Recessive
rs6785049	1.09	0.85–1.38	1.13	0.85–1.49	0.51	Overdominant
rs2276707	1.14	0.88–1.48	1.21	0.90–1.64	0.31	Dominant
rs3814057	1.14	0.91–1.44	1.19	0.92–1.55	0.26	Log-additive

95% CI, 95% confidence interval; OR(adj), odds ratio adjusted for age, gender and smoking; OR(cr), unadjusted odds ratio; p-value, level of significance after correction for multiple testing was set at p = 0.006.

haplotype is composed of the minor alleles rs3814055-T and rs6785049-G, both of which are linked to susceptibility to inflammatory bowel disease. Consistent with the current findings, a significant association was observed for a haplotype composed of the minor rs3814055-T allele with extensive ulcerative colitis in a Spanish cohort (25). Moreover, the rare allele, rs3814055-T, tended to be more prevalent among patients of Spanish and German descent with inflammatory bowel disease (7,25). Contradictory results have been obtained from an Irish sample, showing a significant association of the wild-type rs3814055-C allele with inflammatory bowel disease (8). The discordance in linkage of the rs3814055 allele with inflammatory bowel disease needs further clarification. The different linkage of alleles previous findings in various samples with inflammatory bowel disease, indicate that the rs3814055 polymorphism appears to be linked to chronic inflammatory conditions; however, no specific biological role can yet be confidently ascribed to this genetic variant. It is probable that the haplotype linked to periodontitis tags to an as-yet untested causal genetic variant within the *NR1I2* (*PXR*) locus. If the association

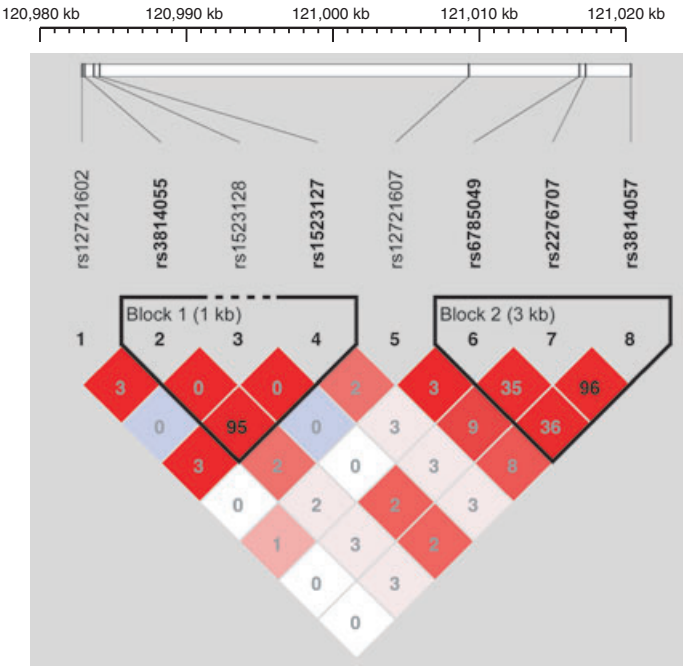

Fig. 1. Distribution of pairwise linkage disequilibrium (LD) for the *NR1I2* (*PXR*) genomic region in the control sample. Numbers given within single cells represent *r*-square values, ranging from 0 (linkage equilibrium) to 1.00 (complete LD). Empty cells correspond to $r^2 = 1.00$. Shading of cells correlates with strength of LD.

Table 4. Frequency estimates for the five-site haplotypes composed of the genotypes for rs12721602, rs3814055, rs1523128, rs12721607 and rs6785049

	Estimated frequencies						
	Periodontitis	Controls	Cumulative	OR(cr) (95% CI)	OR(adj) (95% CI)	*p*-value	*p*-value (permut.)
G-C-G-G-A	0.4128	0.4318	0.4252	1.00	1.00	NA	NA
G-T-G-G-A	0.2108	0.1905	0.6228	0.86 (0.65–1.13)	0.86 (0.63–1.19)	0.39	0.89
G-C-G-G-G	0.1787	0.1877	0.8076	1.00 (0.75–1.32)	0.88 (0.63–1.23)	0.45	0.94
G-T-G-G-G	0.1332	0.1458	0.9489	1.04 (0.78–1.38)	1.10 (0.79–1.54)	0.60	0.98
G-T-G-A-G	0.0307	0.0148	0.9691	0.46 (0.25–0.84)	0.46 (0.23–0.92)	0.011	0.031
A-T-G-G-G	0.0222	0.0166	0.9876	0.73 (0.39–1.38)	0.66 (0.33–1.34)	0.37	0.88

Association analysis was performed for the haplotypes with an estimated frequency of ≥ 1%.
95% CI, 95% confidence interval; NA, not available; OR(adj), odds ratio adjusted for age, gender and smoking; OR(cr), unadjusted odds ratio; *p*-value (permut.), *p*-value after permutation with 100,000 iterations.

found in our study sample can in fact be successfully replicated in independent periodontitis samples, future studies should be directed towards the fine mapping and sequencing of the *NR1I2* (*PXR*) gene in order to elucidate the causal genetic variant.

Considering our results under functional aspects, one of the major environmental risk factors for periodontitis is tobacco use (26). Many tobacco-derived chemicals, for example, nitrosamines or polycyclic aromatic hydrocarbons, are metabolized by cytochrome P450 (CYP) enzymes (27,28). The expression of *CYP* genes is regulated by PXR. Moreover, PXR functions not only as a xenobiotic sensor regulating the downstream expression of genes encoding drug-metabolizing enzymes, but is also part of a complex of intestinal sensing systems that governs the host answer to infections and inflammatory events on or within the intestinal mucosa (29,30). Activation of NF-κB inhibits the function of PXR, thereby causing a reduced expression of its target genes, and conversely, inhibition of NF-κB leads to a higher PXR activity. Hence, apart from xenobiotic metabolism, PXR plays a significant role as a negative mediator of inflammation and immunity. Hence, the possible association of the *NR1I2* (*PXR*) gene variants with chronic periodontitis seems plausible given the dual role of PXR in the expression of *CYP* genes and the regulation of inflammation. A recent study on a Korean cohort in fact suggested that a variant in the *CYP1A1* gene significantly influences the

individual's susceptibility to periodontitis (27).

In conclusion, our study provides evidence for minor contribution of a rare haplotype of the *NR1I2* (*PXR*) gene to the individual's susceptibility for periodontitis. Taking into account the borderline significance and the small effect size of the tested variant, further confirmation of the association is essential.

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Conflict of interest

The authors declare that there is no conflict of interest.

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